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Food and Cosmetics Toxicology

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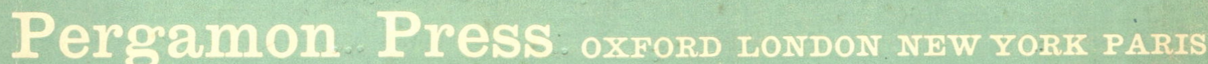

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*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

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Research Section

STUDIES ON THE FATE OF QUINOLINE YELLOW IN THE RAT

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(*Received 24 July 1978*)

Abstract—The uptake, fate and excretion of quinoline yellow in the rat were studied. In the isolated perfused liver 70% of the dose was excreted into the bile within 3 hr. Similar results were obtained after intravenous injection into anaesthetized animals. When given by gavage, only about 1% was excreted in bile and urine within 4–5 hr. Quinoline yellow administered orally to rats was quantitatively excreted, the excretion being mainly faecal. Administration of quinoline yellow did not affect the activities of two microsomal enzymes, *N*-aminopyrine demethylase and aniline hydroxylase. It is concluded that very little quinoline yellow is absorbed from the gastro-intestinal tract of the rat. No evidence of metabolism was obtained.

INTRODUCTION

Quinoline yellow is used as a colouring in sweets and soft drinks. It has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (1975) and given a temporary acceptable daily intake (ADI) of 0–0·5 mg/kg body weight, on the basis of results from acute and chronic toxicity studies. However, as pointed out by the Committee, little is known about its fate in the body. The following experiments were undertaken to gain some insight into the absorption, excretion and possible metabolism of quinoline yellow in the rat.

EXPERIMENTAL

Male and female Sprague–Dawley rats, weighing 200–300 g, were used. They were SPF-bred and maintained in a special animal unit with strict hygienic barriers before the experiments.

Animals used for studying biliary excretion in the isolated perfused liver were anaesthetized with diethyl ether. The common bile duct was cannulated and the liver was dissected and connected to an artificial system containing 10–12% human erythrocytes and 2·5% bovine albumin in a physiological salt solution (for details see Wahlström & Blennow, 1978). The volume of the perfusion fluid was 75 ml, the temperature was 37°C and the pH was 7·4. Quinoline yellow obtained from Merck AG, Darmstadt, BRD, was added dissolved in 0·9% NaCl. Bile and perfusion fluid were sampled at regular intervals during the experiment. The perfusion usually lasted 3–4 hr.

Animals used for studying biliary and urinary excretion after iv or oral administration were anaesthetized with Nembutal (Abbott Laboratories, Chicago, IL, USA; 50 mg/kg body weight). The common bile-duct was cannulated and bile was collected for 4–5 hr. Urine was collected at the end of the experiment. Blood samples were taken from the jugular vein during the experiment. Quinoline yellow dissolved in 0·9% NaCl was administered either iv into the femoral vein or orally by stomach tube.

Animals used for metabolic experiments were placed in individual metabolic cages (Tecniplast, Milan, Italy) and fed water and ground standard feed (Astra-Ewos, Södertälje) *ad lib.* After 24 hr in the cage, quinoline yellow dissolved in 0·9% NaCl, was administered by stomach tube. Urine and faeces were collected after 24, 48 and 72 hr in the cage. Faeces were freeze-dried before extraction of the quinoline yellow.

In some experiments the activities of *N*-aminopyrine demethylase and aniline hydroxylase in liver microsomes were measured. Microsomes were isolated by homogenizing the liver in an isotonic phosphate buffer, pH 7.4, centrifuging the homogenate at 10,000 *g* for 20 min, discarding the pellet and recentrifuging at 105,000 *g* for 60 min. The microsomal pellet was resuspended in buffered isotonic sucrose to a concentration corresponding to 0·5 g liver/ml. The activities of *N*-aminopyrine demethylase and aniline hydroxylase were then determined by standard methods (La Du, Mandel & Way, 1971).

Quinoline yellow in bile, plasma and urine was determined after extraction with 0·1 M-HCl at 418 nm on a Beckman DB-25 spectrophotometer, and in faeces, after repeated extraction with 70% ethanol, at 420 nm on the same instrument. The spectra of quinoline yellow extracted from bile, plasma, urine and faeces were checked against those of quinoline yellow standard solutions in 0·1 M-HCl and 70% ethanol respectively.

RESULTS AND DISCUSSION

After addition of quinoline yellow (25 mg/100 ml perfusion medium) to the isolated perfused liver, about 70% of the administered dose was excreted into

Table 1. *Recovery of quinoline yellow in bile, urine, blood and faeces after isolated liver perfusion, iv injection to anaesthetized animals, oral administration to anaesthetized animals, or oral administration to conscious animals*

Type of experiment (no. of animals)	Duration (hr)	Recovery (% of administered dose) in . . . Bile	Urine	Blood	Faeces	Total recovery
Perfused isolated liver (6)	3	67 ± 6	—	26 ± 4	—	93 ± 3
Intravenous injection (8)	4	59 ± 9	19 ± 4	ND	—	78 ± 10
Oral administration (5) (anaesthetized)	4	0·85 ± 0·38	0·07 ± 0·02	ND	—	0·92 ± 0·38
Oral administration (5) (conscious)	48	—	2·1 ± 1·5	—	97 ± 3	99 ± 3

ND = Not detectable

Values are means ± SEM.

the bile within 3 hr (Table 1 & Fig. 1a). The excretion was rapid and the maximum concentration of the colouring in the bile, 30–50 mg/ml, was found after about 30 min (Fig. 1a). The concentration of quinoline yellow in the perfusion fluid decreased concomitantly with increasing biliary excretion (Fig. 1b), and the recovery of quinoline yellow from perfusion fluid and bile was almost quantitative (about 95%), showing that quinoline yellow was not significantly metabolized. Quinoline yellow did not affect blood flow through the perfused liver, but the bile flow was always slightly increased during the first 30 min after administration (Fig. 1c).

In the anaesthetized animal when the substance was injected iv in a dose calculated to give approximately the same concentrations in the blood as in the isolated liver-perfusion system (Table 1), the excretion of quinoline yellow was quantitatively similar to that during liver perfusion. About 80% of the administered dose was excreted within 4 hr, about 60% in the bile and 20% in the urine. It seems likely that the 20% not recovered was located in various organs, especially the liver and kidneys. In these experiments quinoline yellow had no effect on bile flow. The concentration of quinoline yellow in the blood after injection fell quickly and the substance could not be detected in plasma after more than 60 min. The maximum biliary concentration of quinoline yellow was lower, 2–7 mg/ml, than during liver perfusion, but the bile flow was three to ten times faster,

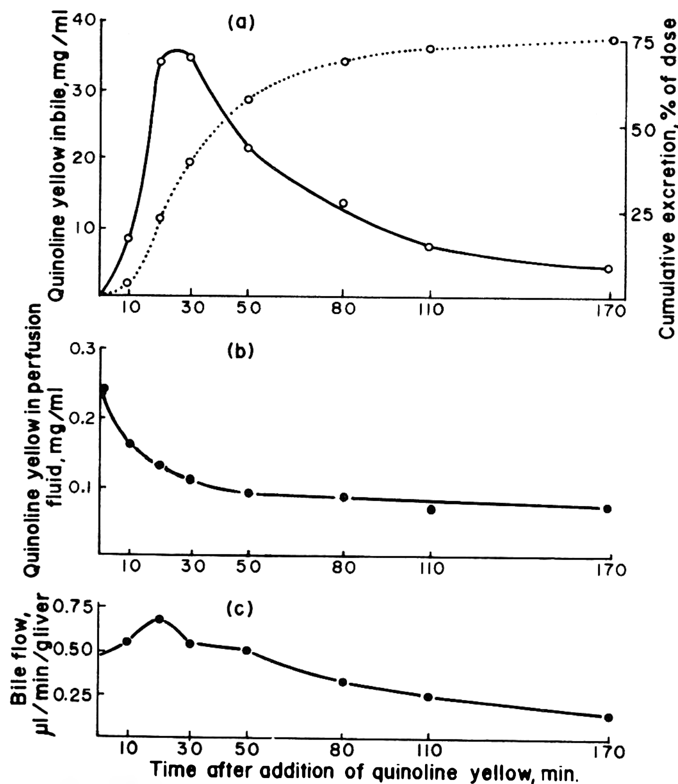

Fig. 1. Excretion of quinoline yellow introduced into an isolated liver-perfusion system at a level of 25 mg/100 ml perfusion medium: (a) concentration of quinoline yellow in bile (——) and accumulated excretion as a percentage of the added dose (· · · · · ·); (b) quinoline yellow in the perfusion fluid; (c) bile flow.

so that the amounts excreted in the two types of experiment were comparable.

On the other hand, when quinoline yellow (1 g/kg body weight) was given by gavage to the anaesthetized animal, only about 1% of the administered dose was recovered from bile and urine after 4-5 hr, showing that very little of the substance is absorbed from the intestine. The main route of excretion of the absorbed quinoline yellow was the bile (about 90%), while only a small amount was found in the urine (Table 1). In these experiments quinoline yellow could not be detected in the blood.

In the overall metabolism experiment about 95% of the administered dose appeared in faeces and about 2% in the urine (Table 1). The excretion of quinoline yellow was essentially complete after 24 hr. The almost quantitative recovery of quinoline yellow in this experiment agrees well with the results from liver perfusion and iv injection as well as with those from oral administration during anaesthesia.

In an *in vitro* test, quinoline yellow (1 mg/ml) was added to a 50% suspension of caecal contents in 0·9% NaCl and incubated at 37°C anaerobically for 24 hr. There was no decrease in quinoline yellow concentration in the suspension, which shows that the substance is not affected by intestinal bacterial known to metabolize azo dyes (Radomski & Mellinger, 1962).

The effect of quinoline yellow on the drug-metabolizing system in the liver microsomes was also tested. The activities of *N*-aminopyrine demethylase and aniline hydroxylase were determined in livers taken from perfusion experiments, from animals that had been injected iv, and in a separate experiment from animals that were given quinoline yellow (1 g/kg body weight) by gavage and killed 24 hr later. There were no differences between the enzyme activities of microsomes from control and treated rats or rat livers in any of these experiments.

In conclusion, we find that quinoline yellow is poorly absorbed from the rat intestine, and what is absorbed is rapidly re-excreted through the bile. With the present methods we could find no evidence of quinoline yellow metabolism in the rat liver or intestine.

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MUTAGENICITY STUDIES OF R-AMINO SALT, A METABOLITE OF AMARANTH (FD & C RED NO. 2), IN MOUSE LYMPHOMA CELLS HETEROZYGOUS AT THE THYMIDINE KINASE LOCUS AND IN THE RAT DOMINANT LETHAL TEST

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(*Received 21 April 1978*)

Abstract—The R-amino salt of amaranth (FD & C Red No. 2) was tested for mutagenicity in the thymidine kinase heterozygous ($TK^{+/-}$) mouse lymphoma assay and in the dominant lethal test. The results from the mouse lymphoma assay showed a dose-related increase in mutation frequency compared with values for the negative controls. Giant cells were present in the cultures after treatment, and a distinct change of colour was observed when the R-amino salt was dissolved in the tissue-culture medium. In the dominant lethal test, the effect of R-amino salt was not statistically significant.

INTRODUCTION

The red food colouring amaranth (FD & C Red No. 2) was officially recognized for use in foods on 13 July 1907 by the Bureau of Chemistry, US Department of Agriculture (USDA, 1907). The dye has since been used in many food items, including soft drinks, dietary supplements and drugs, but in 1976 it was removed from the US market (*Federal Register* 1976, **41** 5823).

Earlier, the Joint FAO/WHO Expert Committee on Food Additives (1966) had reviewed the available toxicology data and determined that amaranth in amounts up to 1·5 mg/kg/day could be consumed safely. Nevertheless, the Committee recommended that long-term studies should be conducted to evaluate the effects of this colouring on reproduction and progeny development. Publications followed in which amaranth, given by gavage to rats, was reported to be embryotoxic (Shtenberg & Gavrilenko, 1970) and to have gonadotoxic effects (Shtenberg & Gavilenko, 1972). Collins & McLaughlin (1972) later confirmed the embryotoxicity of amaranth given by gavage to rats. However, in later studies in which amaranth was given to rats in the diet (Collins, Keeler, Black & Ruggles, 1975) or by gavage (Khera, Przybylski & McKinley, 1974), embryotoxicity was not observed.

Amaranth is metabolized by intestinal bacteria to sodium naphthionate (sodium α-naphthylamine-4-sulphonate) and the R-amino salt (1-amino-2-naphthol-3,6-disulphonic acid sodium salt) (Radomski & Mellinger, 1962; Roxon, Ryan & Wright, 1967). Both these metabolites are absorbed from the gut (Pritchard, Holmes & Kirschman, 1976). Collins & McLaughlin (1973) performed additional studies to determine whether the embryotoxic effects of amaranth could be due to the presence of these metabolites or of an intermediate, the R salt (2-naphthol-3,6-disulphonic acid sodium salt). Both sodium naphthionate and the R salt had an embryotoxic effect, but the R-amino salt did not. However, when the R-amino salt was tested cytogenetically in rats, chromatid breaks were observed in bone-marrow cells (F. M. Moreland, Food and Drug Administration, personal communication 1977).

In an effort to assess the significance of the cytogenetic effect observed in rat bone marrow, the R-amino salt was tested for mutagenic effects in the dominant lethal assay. This test could provide data correlating with the somatic bone-marrow data, since dominant lethality has been shown to be highly correlated with chromosome aberrations in germinal cells (Brewen & Payne, 1976; Hitotsumachi & Kukuchi, 1977). The L5178Y thymidine kinase heterozygous ($TK^{+/-}$) mouse lymphoma assay was also used to assess the potential of the R-amino salt to produce gene mutations in an *in vitro* mammalian cell system.

EXPERIMENTAL

Chemicals. The R-amino salt (97% pure) was synthesized by the Food and Drug Administration, Division of Colors and Cosmetics Technology. Its structure is shown in Fig. 1. The compound was stored in coloured glass bottles in a nitrogen atmosphere at −10°C to prevent oxidation. Solutions, in Fischer's medium for the mouse lymphoma assay and in dimethylsulphoxide (DMSO) for the dominant lethal test, were prepared immediately before use. The positive control compound for the mouse lymphoma assay was ethyl methanesulphonate (EMS), obtained from Eastman Organics, Rochester, NY. The positive control for the dominant lethal test, triethylenemelamine (TEM), was obtained from Lederle Laboratories, Pearl River, NY.

Mouse lymphoma cell assay

Cells. L5178Y mouse lymphoma cells, which are heterozygous for the thymidine kinase locus, were

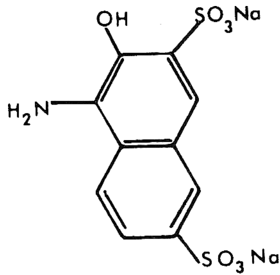

Fig. 1. Structural formula of R-amino salt.

obtained from Dr. Donald Clive and were designated $TK^{+/-}$ 3.7.2. $TK^{+/-}$ cells are sensitive to bromodeoxyuridine (BUdR) and are killed in its presence, whereas $TK^{-/-}$ mutant cells grow in the presence of BUdR; this provides a selective basis for detecting induced mutations at the thymidine kinase locus. Stock cultures were maintained in supplemented Fischer's medium (F_{10}) and were grown in suspension in 300-ml flat-bottom, screw-cap Erlenmyer flasks in a gyroshaker at a speed of 200 revs/min (Palmer, 1978). Stock cultures were cleansed to reduce the background $TK^{-/-}$ mutants by the overnight exposure of cells growing in log phase to THMG medium, containing thymidine (3 μg/ml), hypoxanthine (5 μg/ml), methotrexate (0·1 μg/ml) and glycine (7·5 μg/ml), according to the method of Clive & Spector (1975). After the THMG treatment, the cells were grown for 24 hr in THG medium (THMG minus methotrexate) before the culture was used for an experiment.

Procedure. The experimental protocol was a modification (Palmer, 1978) of the method described by Clive & Spector (1975). Six million cells contained in 9 ml of F_{10} were placed in 50-ml plastics conical centrifuge tubes, and the R-amino salt contained in 1 ml of Fischer's medium was added. The concentrations to be used were determined by viability cloning in soft agar immediately after a 4-hr chemical exposure. Dose levels of 25, 50 and 75 μg/ml were chosen to represent low, intermediate and high doses, respectively, and duplicate or triplicate cultures were set for each concentration. Solvent controls (1 ml of Fischer's medium) and positive controls (EMS at 155 μg/ml) were included with each experiment. The cultures were placed in a roller drum for a 4-hr exposure period. The cells were then washed twice with F_{10} and resuspended in 15 ml of F_{10} for a 72-hr expression period. Cell counts were made after the washings and at 24-hr intervals during the expression period, and each culture was reduced at a cell density of 3×10^5 cells/ml. At the end of the expression period, 3×10^6 cells were pelleted and resuspended in cloning medium (Palmer, 1978). Cultures were serially diluted, treated with BUdR, and cloned in soft agar (Chu & Fisher, 1968) to determine the number of BUdR-resistant cells and the relative viability of each culture (Palmer, 1978). At the end of 10 days, the clones were scored and mutation frequencies were calculated.

Dominant lethal study

Animals. Holtzman albino rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. The males were 9 wk old and had an average body weight of 311 g at the initiation of treatment. The females were 11 wk old and had an average body weight of 245 g at the time of mating.

Dose-level determination. Appropriate dose levels for the test were estimated from a subacute toxicity study conducted with doses ranging from 75 to 300 mg/kg body weight/day. The compound was administered to three rats at each dose level by ip injection daily for a maximum of 5 days. All rats treated with doses of 200 mg/kg or more died within a few days; therefore 150 mg/kg/day was selected as the highest dose for testing. The medium and low doses were 75 and 37·5 mg/kg/day, respectively.

Protocol. Solutions of the R-amino salt in DMSO were prepared daily and administered to rats by ip injection within 1 hr of preparation. Stock solutions of TEM in distilled water, prepared weekly, were stored at −20°C, and aliquots were diluted daily with distilled water. Five groups, each of 15 male rats, were given either DMSO (the negative control compound), TEM (the positive control compound) in doses of 0·035 mg/kg/day, or the R-amino salt in doses of 37·5, 75 or 150 mg/kg/day, the injection volumes for DMSO and each solution being 1 ml/kg body weight. The rats were treated on 5 days/wk for 10 wk as described by Green, Moreland & Flamm (1977), and after the final injection each male was immediately caged with two females per week for 2 successive weeks. The females were killed 17 days after the first day with the male, and the numbers of corpora lutea and live and dead implantations were counted.

The following indexes were evaluated (Green, Zeiger, Palmer, Springer & Legator, 1976). Numbers of corpora lutea per pregnant female and total implantations per pregnant female were analysed by the Student's t test. Pre-implantation losses per pregnant female and dead implantations per pregnant female were analysed by the t test after transformation by the Freeman-Tukey square-root method, and dead implantations per total implantations were analysed by the t test after transformation by the Freeman-Tukey arc-sine method. Proportions of females with one or more dead implantations and with two or more dead implantations were analysed by the chi-square test.

RESULTS

Mouse lymphoma cell assay

Table 1 presents the data obtained from tests of the R-amino salt by the $TK^{+/-}$ L5178Y mouse lymphoma forward mutational assay. The results are given as averages of three replicates for experiment 1 and two replicates for experiment 2. The increases in mutation frequency of cells treated with the R-amino salt were dose-related and results were comparable in the two experiments, strengthening the conclusion of a positive response. EMS, used at a level that produced little reduction in viability, gave a definite positive response, indicating that the system can detect mutagenic compounds when other signs of toxicity are less evident.

Other observations made during the experiments were the presence of giant cells after treatment with the R-amino salt and a rapid change in the colour

Table 1. *Effects of R-amino salt in the TK+/− L5178Y mouse lymphoma cell assay*

Compound	Concn (μg/ml)	Survival (%)	Mutants/ 10^5 survivors	Induced mutation frequency*
		Experiment no. 1†		
F_0	0	100	4·29	—
R-amino salt	25	52·8	9·37	5·08
	50	4·8	21·90	17·61
EMS	155	52·7	15·91	11·62
		Experiment no. 2‡		
F_0	0	100	5·65	—
R-amino salt	25	77·8	7·54	1·89
	50	3·2	44·77	39·12
	75	0·3	117·54	111·89
EMS	155	94·2	16·73	11·08

F_0 = Fischer's medium without additives EMS = Ethyl methanesulphonate
* The background spontaneous mutation frequency was subtracted from the induced mutation frequency observed.
† Values are the means of results obtained from three cultures per dose level.
‡ Values are the means of results obtained from two cultures per dose level.

of the chemical solution, indicating a possible change in the test compound. The number of giant cells increased with the concentration of the chemical so that at the highest dose level the majority of the cells were giant. The change from red to light brown was observed immediately after the R-amino salt was diluted in unsupplemented Fischer's medium.

Dominant lethal study

Effects of 10-wk treatment on weight gain and mortality. Within 2 days, the R-amino salt at 150 mg/kg/day had caused ten deaths among 15 treated rats. Because of this unexpected toxicity, a group of ten rats was added to the study and the 150-mg/kg group was eliminated. Four of the additional ten rats, which were treated at a dose level of 120 mg/kg/day, died (Table 2); these deaths occurred during treatment wk 2, 7, 8 and 10. A few deaths, however, also occurred in the control and other treated groups. In addition, the R-amino salt appeared to cause a dose-related inhibition of body-weight gain during the 10 wk of treatment (Table 2).

Dominant lethal effects. Table 3 shows the results of the dominant lethal study. The positive control compound, TEM, produced a statistically significant level of dominant lethality. The R-amino salt at daily dose levels of 37·5, 75 and 120 mg/kg/ produced variable effects, depending on the indexes analysed.

The numbers of corpora lutea per pregnant female were significantly increased ($P < 0·01$) in treated animals that were mated during wk 1; however the biological importance of this change is not clear. The number of dead implantations per pregnant female, an index of dominant lethality, showed some indication of a dose-related increase for the wk-1 mating but the increases were statistically insignificant; the number (1·2) at the high dose level was identical to that for the TEM-treated group, but the six dead implantations at the high dose level occurred in one female whereas the 12 dead implantations in the TEM-treated group were spread over eight females. Therefore the decreased number of pregnant females and the large variability between females at the high dose level both contributed to the non-significance of this value. Values for all other indexes were comparable to those of the DMSO control group.

DISCUSSION

Data from the TK+/− L5178Y mouse lymphoma tests provide evidence that a mutagenic response is induced by the R-amino salt. The dose-related response observed in the first experiment was reproducible in the second. Correlation of the actual values between the two experiments was not good, but the induced mutation frequency showed a dose-related increase in each experiment. Although the low survival and high mutation frequency could indicate selection, the actual numbers of mutant clones observed in the

Table 2. *Survival and mean body-weight gains in male rats treated with R-amino salt for 10 wk*

Treatment	Dose level (mg/kg/day)	No. of males/group		Mean body-weight gain	
		Initial	Final	g	%
DMSO	—	15	11	173	100
R-amino salt	37·5	15	13	153	88
	75	15	11	135	78
	120	10	6	111	64
TEM	0·035	15	13	169	98

DMSO = Dimethylsulphoxide TEM = Triethylenemelamine

Table 3. *Reproduction indices for matings with male Holtzman rats treated with R-amino salt for 10 wk in a dominant lethal study*

Parameter		Reproduction data following matings with males treated with									
		DMSO (1 ml/kg/day)		R-amino salt in doses (mg/kg/day) of						TEM (0·035 mg/kg/day)	
				37·5		75		120			
	Mating wk...	1	2	1	2	1	2	1	2	1	2
Total females mated		22	22	26	26	22	22	12	12	26	26
No. of pregnant females		14	18	19	23	15	20	5	9	10	7
Pregnancy rate (%)		64	82	73	89	68	91	42	75	38	27
No. of corpora lutea/pregnancy		11·9	13·8	12·2	13·5	13·7	12·7	15·4**	11·1	5·7**	5·9**
Pre-implantation losses/pregnancy		1·00	0·28	0·37	0·17	0·60	0·20	0·40	1·11	4·10**	3·86**
Total implantations/pregnancy		12·9	13·6	11·8	13·3	14·3	12·9	15·0	10·0	1·6**	2·0**
Dead implantations/pregnancy		0·50	0·72	0·84	0·57	1·07	1·10	1·20	1·00	1·20**	2·00**
Dead implantations/total implantations		0·04	0·05	0·07	0·04	0·07	0·09	0·08	0·10	0·75*	1·00
Pregnancies with at least one dead implantation:											
No.		7	9	10	12	9	11	1	5	8	7
Proportion of total pregnancies		0·50	0·50	0·53	0·52	0·60	0·55	0·20	0·56	0·80	1·00*
Pregnancies with two or more dead implantations:											
No.		0	3	5	1	3	5	1	3	4	4
Proportion of total pregnancies		0	0·17	0·26	0·04	0·20	0·25	0·20	0·33	0·40*	0·57

DMSO = Dimethylsulphoxide TEM = Triethylenemelamine

Values marked with asterisks differ significantly from the control value: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

treated cultures were high enough to make this possibility unlikely. These data, together with the observation of giant cells in treated cultures, may indicate the transforming ability of the R-amino salt. Giant cells are associated with the loss of formation of microtubules, which is a characteristic of transformed cells (Brinkley, Fuller & Highfield, 1975). Therefore *in vitro* transformation studies would be helpful in assessing the potential hazard of R-amino salt. The dominant lethal data indicate that this compound did not induce a detectable level of dominant lethality under the test conditions used.

One factor that must be considered is the change in the colour of the stock solution when the compound was dissolved in Fischer's medium. This change could be the result of oxidation of the R-amino salt to an *o*-quinone structure (J. Dantzman, Food and Drug Administration, personal communication 1978), which may be the active component in the mouse lymphoma system. Such a colour change was not noticed when the compound was dissolved in DMSO and administered to the rats within 1 hr of preparation. The fate of the R-amino salt *in vivo* is not known. It is very likely that again an oxidation process takes place, perhaps more completely, resulting in a less mutagenic end product *in vivo*. The metabolic fate of the R-amino salt may be partly responsible for the difference in responses obtained with the mouse lymphoma cell assay and with the rat dominant lethal test.

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TWO-YEAR ORAL TOXICITY AND MULTIGENERATION STUDIES IN RATS ON TWO CHEMICALLY MODIFIED MAIZE STARCHES

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Abstract—Two modified maize starches, acetylated distarch adipate and acetylated distarch glycerol, each at a level of 62% in a cooked diet, were fed to groups of Sprague–Dawley-derived rats throughout a 2-yr oral toxicity study and multigeneration study (three generations over approximately 2 yr). Unmodified maize starch (62%) in a cooked diet was fed to controls. Behaviour and food consumption were not significantly altered by the treatment. Body weights of both groups of treated animals, and particularly of the females, were somewhat lower than those of the controls from month 6 of treatment, but femur measurements indicated no accompanying differences in skeletal growth and, at autopsy, control rats contained markedly greater adipose deposits than were found in the treated rats of either group. These findings limit the significance of the body-weight differences. Haematology, serum biochemical analyses, bacteriological examinations and organ-weight determinations showed no significant differences of pathological interest between control and treated animals. Over the 2-yr test period the death rate was slightly lower in both groups fed modified starches than in the control group. Histological examination of the main organs did not reveal any significant differences between control and treated groups; all lesions and tumours were distributed randomly. The absence of treatment-related renal changes contrasted with the results of de Groot *et al.* (*Fd Cosmet. Toxicol.* 1974, **12**, 651) who fed other modified starches to Wistar-derived rats. The multigeneration study revealed no effects on fertility, litter size or embryonic or pre-weaning mortality and histological examinations of the F_{3b} generation showed no treatment-related anomalies. Thus no distinct effect of toxicological importance resulted from feeding rats with either of these modified maize starches at dietary levels of 62% for 2 yr.

INTRODUCTION

The two modified maize starches considered in this study were acetylated distarch glycerol and acetylated distarch adipate. Derived from natural starches by chemical treatment, they are used as thickeners, binders and stabilizers in prepared foods such as canned goods, baked goods, frozen foods, salad dressings and fruit-pie fillings. The amounts added to foods are self-limiting, since with levels above about 10% the degree of binding or thickening becomes excessive.

There are few published reports of toxicity trials of these two modified starches, and in particular there is a lack of information on long-term studies. A short-term feeding study of acetylated distarch glycerol in miniature pigs was reported by Anderson, Filer, Fomon, Andersen, Jensen & Rogers (1974) and unpublished reports have been summarized by the Joint FAO/WHO Expert Committee on Food Additives (1972). In the work reported here, the modified maize starches were fed at dietary levels of 62% in a 2-yr oral toxicity study and in a concurrently performed three-generation study, which was started with animals picked randomly from the oral toxicity study. The control groups received diet containing 62% unmodified maize starch.

EXPERIMENTAL

Test materials. Acetylated distarch glycerol (EEC no. 1423) was prepared by modification of maize starch with epichlorhydrin to a maximum of 0·3% and acetic anhydride to a maximum of 8%; the acetyl groups introduced did not exceed 2·5% and the residual level of epichlorhydrin and/or glycerol mono- and dichlorhydrins was below 1 mg/kg (the limit of detection with the analytical technique used). For acetylated distarch adipate (EEC no. 1422), modification was carried out with acetic anhydride and adipic acid, the latter acting as a cross-linking agent. The maximum number of acetyl groups introduced was again 2·5% and the maximum number of adipic cross-links was 1 in about 1000 glucopyranose units, introducing not more than 0·09% adipyl groups into the starch.

Animals and diets. OFA (Sprague–Dawley-derived) rats bred under specified-pathogen-free conditions were aged 4–5 wk at the start of the study, throughout which they were maintained under conventional conditions. Air was renewed in the animal house

10 times/hr and kept at 21 ± 1°C and a relative humidity of 40–60%. During the oral toxicity study, rats were kept in groups of five, in cages 43 × 30 × 18 cm; during gestation and suckling periods in the multigeneration study, the females were housed singly in cages 43 × 22·5 × 18 cm.

Three groups of 40 male and 40 female rats were fed a cooked diet based on a feed habitually used for rats but with a higher vitamin content since it was heat-treated. The basal diet consisting of maize starch (620 g), molasses bran (30 g), soya-bean cake (140 g), fish meal (110 g) and powdered blood (50 g) was supplemented with the following minerals and vitamins: yeast (30 g), calcium phosphate (5 g), sodium chloride (5 g), seaweed meal (5 g), manganese sulphate (5 mg), copper sulphate (0·4 mg), iron sulphate (1 mg), cobalt sulphate (0·05 mg), vitamins B_1 (5 mg) and B_2 (14 mg), nicotinic acid (48 mg), calcium pantothenate (30 mg), vitamins B_6 (4 mg), K_3 (10 mg), A (16,000 IU), D_3 (4000 IU) and E (100 mg) and methionine (500 mg). Each group received a calorifically identical diet containing 62% starch, which provided practically all of the animals' carbohydrate intake and consisted of either unmodified maize starch (group A; control), acetylated distarch glycerol (group B) or acetylated distarch adipate (group C). A paste formed by mixing 30 parts of powdered test or control diet with 70 parts of water at 70–72°C was poured into plastics bags, which were sealed and placed in boiling water for 1·5 hr. Feeds were prepared weekly and kept in cold store and, to prevent spoilage, were offered to all groups every Monday, Wednesday and Friday, in rations of 60 g/rat/day, equivalent to 42 g dry weight/rat/day.

Experimental design and conduct

Oral toxicity study. Of the 40 males and 40 females in each group, ten males and ten females were killed for autopsy and histological examination after 3 months. The remaining 30 males and 30 females in each group were treated for 24 months. All the rats were weighed individually twice a week for the first 3 months, weekly for the next 3 months and twice a month from 6 months. Food consumption was measured for each cage of five rats, twice a week for 6 months and then monthly for the next 12 months only.

Haematological examinations (blood cell counts using a Coulter counter, differential leucocyte counts with May-Grünwald–Giemsa staining, and determinations of haemoglobin and packed cell volume) were carried out on five males and five females from each group initially and after 3 months and on ten males and ten females from each group at months 12 and 24. A bone-marrow differential cell count (using May-Grünwald–Giemsa staining) was conducted on five males and five females from each group at month 24. Serum analyses carried out at month 3 (five males and five females/group) and at months 12 and 24 (ten males and ten females/group) covered levels of sodium and potassium, chlorides (at months 12 and 24 only), calcium, phosphorus, glucose, blood-urea nitrogen, uric acid (at month 3 only), cholesterol, total protein, bilirubin, alkaline phosphatases, glutamic–oxalacetic and glutamic–pyruvic transaminases (SGOT and SGPT; at months 12 and 24 only) and creatinine (at month 3 only), as well as electrophoresis measurements. [Full details of the analytical methods are available from the authors.] Intestinal microflora were examined quantitatively and qualitatively at months 3 and 24 in five rats/group using the technique of Perrot (1976), and the findings were compared with values established for the control group at the start of the study.

Half of the 20 animals from each group autopsied after 3 months were also examined histologically. At month 24 all surviving rats were killed for autopsy and histological examination. Animals dying during the course of the trial or killed because of poor clinical condition were also examined, except where autolysis was too advanced. In all cases the heart, liver, spleen, kidneys, adrenals and gonads were weighed and at month 24 the brain, pituitary and thyroid were also weighed. All these organs and the pancreas, stomach, duodenum, jejunum, colon, bladder, submaxillary salivary glands and lymph nodes were examined histologically. The femur was removed for measurement.

Multigeneration study. The parent animals (F_0) for this study were ten males and ten females from each of the groups participating in the oral toxicity study. After two matings, producing the F_{1a} and F_{1b} generations, the parents were returned to the oral toxicity trial. The F_{1a} generation was killed and autopsied at weaning. The F_{1b} generation was also killed except for ten males and ten females taken at random from each group. These were mated twice to produce the F_{2a} and F_{2b} generations and were then killed and autopsied. The procedure followed with the F_2 generations was the same as that for the F_1 groups, the resulting F_{3a} and F_{3b} generations being killed and autopsied at weaning and 6 wk after weaning respectively. The F_{3b} generation was also examined histopathologically.

There was a 12–15-wk interval between first and second pairings and all mating was monogamous, one male and one female from the same group being placed together for 21 days. Records were kept of the number of pups (live and stillborn) in each litter, the total number of pups/group, and hence the mean number/female, the litter weights at days 1, 5, 15 and 21 (weaning), the percentage mortality during suckling, compared with the live young at day 1, the sex ratio of litters at day 21, and the mean duration between pairing and parturition. Since the gestation period is 21 days and the oestrous cycle 5 days, the average duration between pairing and parturition was 25–30 days.

RESULTS

Oral toxicity study

No behavioural abnormalities were observed during the study. By the end of the trial 48% of the rats in group A (controls), 40% in group B and 40% in group C had died or been killed because of their poor clinical condition, as in the case of females presenting mammary growths. This level of mortality was to be expected in ageing rats. Growths were noted macroscopically in all groups, but in the females they were less frequent and appeared later in the treated groups than in the control group. Thus the incidence

Table 1. *Mean terminal body weights of rats fed cooked feed containing 62% acetylated distarch glycerol or acetylated distarch adipate for 2 yr*

Group*	No. of rats	Mean terminal body weight: Weight (g)	Difference from control group (%)
		Males	
A	19	598	
B	21	525	−12·2
C	14	522	−12·7
		All females	
A	12	421	
B	16	346	−17·8
C	21	335	−20·4
		Females excluding those with large tumours	
A	7	408	
B	10	339	−16·9
C	14	337	−17·4

* Group A, control; B, fed acetylated distarch glycerol (starch no. 1423); C, fed acetylated distarch adipate (starch no. 1422).

in groups A, B and C, respectively, was 10, 6·7 and 6·7% in the males and 73·3, 50 and 50% in the females. This helps to explain the fewer deaths in treated groups since it was often necessary to kill females with growths large enough to restrict movement or feeding.

Body weights were almost identical in all the groups up to 3 months. After 6 months the treated animals weighed less than the controls, 4 and 2·7% less for the males of groups B and C respectively and 9·2 and 12% less for the females. By month 24, the differences were more marked (Table 1) particularly in group C females, which weighed 20·4% less than the controls. The body-weight differences were exaggerated in the females by the presence of large tumours in some animals. Eliminating such animals from the calculations gave a maximum difference from controls of 17·4% for group C females (Table 1).

Body-weight differences were not due to a diminished intake of modified starches since food consumption of all three diets was comparable. Skeletal growth, as indicated by femur measurements at autopsy, was also comparable in all groups. However, it was noted at autopsy that adipose deposits were much less extensive in the treated rats than in the controls. Qualitative and quantitative analyses of the intestinal microflora did not reveal any significant differences between the groups. Organisms isolated included Lactobacilli, Streptococci, Staphylococci, Enterobacteria, Escherichia species and Bacilli in quantities normally encountered in SPF rats kept under conventional conditions. The technique used, however, was not suitable for the assessment of strictly anaerobic strains.

All variations in the haematology studies* were within normal limits. There were statistically significant differences between the treated and control rats in some of the serum biochemistry parameters* but all the values were within normal limits with the exception of SGOT at month 24 and none of the differences were considered to be pathologically significant.

Autopsies at 3 months failed to reveal any abnormalities. The mean liver weight of group B rats (given acetylated distarch glycerol) was lower than that of group A but the difference was not significant when the weights were expressed relative to body weight. Autopsies at month 24 were conducted on all surviving animals (31 in group A, 36 in group B and 35 in group C). Gross findings in these rats were those to be expected in ageing animals of this strain. The distribution of mammary tumours in these animals is shown in Table 2. There were fewer in treated than in control rats. Organ weights showed some intergroup variations that reached statistical significance, but the differences rarely occurred in both the absolute and relative values and since no treatment-related changes were noted microscopically the differences were regarded as incidental.

* Full details of these results are available from the authors.

Table 2. *Incidence of mammary tumours in rats surviving at the end of the 2-yr feeding study of modified maize starches*

Group*	No. of females autopsied	No. with mammary tumours	Percentage affected
A	12	5	41·7
B	15	5	33·3
C	21	7	33·3

* Group A, control; B, fed acetylated distarch glycerol; C, fed acetylated distarch adipate.

Table 3. *Incidence and types of tumours in all rats examined histologically (surviving rats and rats killed during the study)*

Site and type of tumours		No. of tumours found					
		Males			Females		
	Group*...	A	B	C	A	B	C
	No. of rats examined...	25	24	21	24	27	28
	Total no. with tumours...	17	20	19	24	22	23
Heart							
Malignant mesothelioma		0	0	0	0	1	0
Lungs							
Pulmonary adenoma (or adenocarcinoma)		1	0	0	0	0	0
Intra-alveolar tumour		0	0	0	0	0	1
Mammary gland							
Adenoma		0	0	0	0	1	3
Fibroadenoma		0	0	0	16	9	12
Adenocarcinoma		0	0	0	2	0	0
Fibroma		0	0	0	0	3	0
Liver							
Vascular polyp (haemangioma)		1	0	0	0	0	0
Mesentery							
Very necrotic fibrosarcoma enveloping digestive and genital organs		0	0	0	0	1	0
Adrenals							
Cortical adenoma (hyperplasia?)		0	1	7	6	5	3
Adenoma of reticular zone, with vascular ectasia		0	1	0	0	0	0
Phaeochromocytoma		2	4	5	1	1	2
Thyroid							
Trabecular adenoma		4	10	6	10	10	5
Minute adenoma (hyperplasia?)		5	8	5	3	2	6
Pituitary							
Adenoma of the anterior lobe		4	5	3	1	2	2
Minute adenoma		3	0	1	2	0	3
Adenoma or adenocarcinoma		0	0	1	0	0	0
Skin							
Keratoacanthoma		2	0	0	0	0	0
Dermatofibroma		1	0	0	0	0	0
Subcutaneous tissue							
Fibroma		0	1	0	0	0	0
Fibrosarcoma		0	0	0	1	0	0
Schwannoma (?) neurofibroma		0	0	1	0	0	0
Zymbal's gland							
Squamous-cell carcinoma		0	0	1	0	0	0
Epididymis							
Undifferentiated embryonic sarcoma		0	0	1	—	—	—
Pleura							
Malignant mesothelioma		0	0	0	0	1	0
Vagina							
Sarcoma		—	—	—	0	0	1
	Total no. of tumours...	23	30	31	42	36	38

* Group A, control; B, fed acetylated distarch glycerol; C, fed acetylated distarch adipate.

There were no histological differences between groups either in relation to non-tumorous lesions or tumours. All tumours were of types normally encountered in rats of this strain. Since survival in treated groups B and C was slightly better than in the control group (A) it was not considered necessary to apply a Peto-type analysis to the results (Peto, 1974). Failure to consider survival can only have biased the results in the direction of making groups B and C seem worse by comparison with group A. Hence the tumour incidence is presented here as a direct comparison between groups (Table 3).

In a study of five modified starches, de Groot, Til, Feron, Dreef-Van der Meulen & Willems (1974) reported the presence of focal hyperplasia of renal papillary and pelvic epithelia, accompanied by calcified patches in the underlying tissues. The renal lesions appeared more frequently in the males of the top-dose groups. In our study of two modified maize starches, neither of which were studied by de Groot *et al.* (1974), hyperplasia of the urothelium, with and without calcification, was also noted but was distributed randomly in all three groups. Particular attention was therefore given to the form and distribution of the renal lesions observed. Table 4 shows the incidence of epithelial hyperplasia and mineral deposits or calcifications. The lesions found in the kidneys were of exactly the same type and of comparable inci-

Table 4. *Incidence of urothelial hyperplasia and calcification in rats fed modified maize starches for 2 yr*

Lesion: Type	Degrees of severity*	No. of rats:	A Males	A Females	B Males	B Females	C Males	C Females
		examined	25	24	24	27	21	28
		aged 2 yr or more†	19	12	21	17	15	20
Hyperplastic urothelium without calcification	+		6	7	5	7	5	8
	+ +		2	1	4	3	1	2
	+ + +		0	0	0	1	0	0
Calcification (uroliths)	+		4	8	9	13	4	8
	+ +		4	4	4	2	5	5
	+ + +		4	0	2	1	6	3
	All		12	12	15	16	15	16

(A, B, C: No. of rats affected in group)

* Slight (+), marked (+ +), or severe (+ + +).
† Fed test or control diet for at least 23 months.

Table 5. *Reproduction data for three generations of rats fed modified maize starches at a dietary level of 62%*

Parent generation	Mating	Group*	Females with litters (%)	Mean no. of pups born/litter	Stillbirths (%)	Preweaning deaths (%)	Sex ratio at weaning (M/F)	Mean body weight (g) at day 1	5	12	21
F_0	1st	A	90	12·2	3·6	2·80	53·4/46·6	6·16	9·92	19·43	33·43
		B	100	11·0	6·4	1·90	47·5/52·5	6·66	10·96	19·30	33·55
		C	100	12·2	3·3	0·85	49·6/50·4	6·16	9·89	17·87	29·06
	2nd	A	100	13·2	8·3	0·83	55·8/44·2	6·10	9·50	17·97	32·03
		B	100	11·2	1·8	1·82	52·8/47·2	6·45	10·03	19·14	33·17
		C	90	12·6	2·7	2·73	51·4/48·6	6·2	9·85	19·33	32·52
F_1	1st	A	90	13·4	0·8	1·67	51·7/48·3	5·80	8·96	17·90	29·50
		B	100	11·1	3·6	0·93	38·7†/61·3	5·94	9·65	19·30	31·10
		C	90	12·8	5·2	1·83	56·1/43·9	5·83	9·60	18·80	29·00
	2nd	A	100	12·3	2·4	2·50	60·7/39·3	6·26	10·00	18·37	33·90
		B	100	12·3	1·6	8·26	44·1/55·9	6·27	10·30	18·87	30·99
		C	89	12·1	3·1	7·45	43·7/56·3	6·20	9·83	18·22	31·50
F_2	1st	A	100	11·5	7·8	5·66	55·0/45·0	6·06	9·94	19·96	36·39
		B	90	10·7	6·3	3.33	56·3/43·7	6·23	10·54	21·41	33·56
		C	80	12·5	6·0	4·26	42·2/57·8	5·73	9·33	19·58	30·62
	2nd	A	90	11·3	1·0	12·89	47·7/52·3	6·16	10·38	21·32	39·63
		B	90	10·8	3·5	14·46	49·3/50·7	6·54	11·08	23·48	38·52
		C	80	12·5	2·0	22·5	57·9/42·1	6·47	10·34	21·62	34·81

* Group A, control; B, fed acetylated distarch glycerol; C, fed acetylated distarch adipate.
† $\chi^2 = 3·85$.

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dence and severity in both of the treated groups and in the control animals, and therefore were not related to the administration of these modified maize starches. Lesions of this type are commonly found in rats of Sprague–Dawley origin (Magnusson & Ramsay, 1971).

Independent pathologists were asked to examine the slides of kidneys resulting from this study. Prof. P. M. Newberne and Dr. F. J. C. Roe concluded that the lesions were not related to the treatment with modified starches. Dr. A. E. McLean's assessment differed a little from that of the authors, and his results have therefore been included in an Appendix. He reported that the frequency and severity of the lesions was slightly greater in the rats fed modified starches but that the differences were significantly greater only in the occurrence of epithelial hyperplasia, a minor lesion compared with pelvic nephrocalcinosis, in the treated females.

Multigeneration study

Table 5 summarizes the results of this study, during which 57 litters were obtained from group A, 57 from group B and 52 from group C. The mean number of live young per litter at birth was 11·82 for group A, 10·77 for group B, and 11·98 for group C. Postnatal mortality (up to day 21) was between 4 and 6%. These data are within normal limits for the strain of rat used. Growth was comparable in all groups. Terminal studies of the F_{3b} generation (including histology of the principal organs) did not reveal evidence of anomalies.

DISCUSSION

The feeding of two modified maize starches, acetylated distarch glycerol and acetylated distarch adipate, to rats for 2 years thus had no demonstrable effect on the behaviour or condition of the animals or on the analyses of haematological parameters, serum biochemistry and intestinal flora. The survival rate was slightly better for treated than for control animals. Organ weights showed no differences of pathological interest. All aspects of reproduction, fertility, litter size, embryonic and pre-weaning mortality and litter growth were comparable in all groups.

Mean body weights were somewhat reduced in treated animals although food consumption was comparable in all groups. However, at autopsy, no significant differences in skeletal growth were indicated by femur measurement and the controls had markedly greater deposits of fat than the treated animals. These two findings may be considered to reduce the overall biological significance of the lower body weights in the treated rats.

The hyperplasia of the kidney urothelium, sometimes accompanied by calcification, was observed in all groups and neither the incidence nor severity is considered to have been related to the administration of the test starches. These results are contrary, therefore, to those of de Groot *et al.* (1974) who found that pelvic nephrocalcinosis occurred more frequently in males of the top-dose groups. However, it must be remembered that these authors worked with different modified starches, a different strain of rat and a different basal diet. The possible influence of the mineral balance of the diet on the incidence of pelvic nephrocalcinosis must not be ignored when studies are compared. The basal diet used in the study reported here (Table 1) had a well-balanced mineral content and probably had little effect on the incidence of pelvic nephrocalcinosis. It is important that the same basal feed was provided for both treated and control groups and that the level of vitamins was deliberately increased because the food was cooked.

Since the renal lesions were not considered to be related to the administration of the modified maize starches, it is concluded that rats may be fed acetylated distarch glycerol or acetylated distarch adipate at dietary levels of 62% for 2 yr without the induction of any distinct effects of toxicological importance.

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APPENDIX

Dr. C. Fisher and Dr. A. E. McLean of the Department of Morbid Anatomy and Laboratory of Toxicology, University College Hospital Medical School, Rayne Institute, London WC1E 6JJ, agreed to examine the kidney slides from this 2-yr feeding study. Their assessment of the kidney lesions differed somewhat from that of the authors, in that they considered the incidence of epithelial hyperplasia to be significantly greater in treated female rats. With the agreement of Dr. Fisher and Dr. McLean, their results are included here.

The lesions were graded for severity from 1 (normal) to 4 and mean scores for each group and sex were calculated:

		No. of rats affected					
		Males			Females		
Lesion	Grade	A	B	C	A	B	C
Epithelial hyperplasia	1	5	4	2	12	3	1
	2	19	19	18	12	23	25
	3	1	0	1	0	1	2
	4	0	1	0	0	0	0
	Mean...	1·84	1·92	1·95	1·50	1·92	2·04
Calcification	1	8	3	5	8	8	5
	2	13	17	10	12	16	17
	3	2	4	5	4	2	6
	4	2	0	1	0	1	0
	Mean...	1·92	2·04	2·09	1·83	1·85	2·04

Dr. Fisher and Dr. McLean concluded: "Exactly the same kinds of changes were found in kidneys of rats fed maize starch as were found in kidneys of animals fed modified starches. The frequency and severity of lesions was slightly greater in the rats fed modified starches (B & C). The differences were not significant in the case of male rats, but for female rats the proportion of rats showing epithelial hyperplasia was greater in the rats fed modified starches."

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THE METABOLISM OF BEEF TALLOW SUCROSE ESTERS IN RAT AND MAN

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Abstract—Metabolic studies in rats with sucrose esters of beef tallow, given in a single oral dose of 5, 50 or 100 mg/kg, indicated that some 80–90% of the sucrose moiety was absorbed from the gastro-intestinal tract. The disposition of radioactivity after the administration of [^{14}C]sucrose tallowate suggested that the esters were hydrolysed before absorption, and no evidence was obtained for the accumulation of the esters in the adipose tissue of rats following repeated daily oral administration of [^{14}C]sucrose tallowate. Analysis of the urine of human volunteers given either sucrose (10 g) or sucrose tallowate (1 g) indicated the presence of only small amounts of sucrose (6–24 ppm). Because of the apparent ease with which the esters are hydrolysed under physiological conditions to sucrose and the corresponding fatty acids, the use of these materials as additives to human foods would not appear to present a significant toxicological hazard.

INTRODUCTION

Sucrose esters of edible fatty acids possess properties that make them particularly suited for use as emulsifying agents in a variety of foods, including confectionery, processed foods and sauces, and they have been approved for such use in several countries. The toxicological status of this group of materials was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (1976) and the group was assigned a temporary acceptable daily intake (ADI) of 0–2·5 mg/kg body weight on the basis of studies that indicated the no-effect-level in rodents to be 10,000 ppm.

The metabolic disposition of the esters does not appear to have been investigated in detail, and since such information may assist the evaluation of the safety of these materials this aspect has been studied in rats and human volunteers with the mono- and di-esters derived from beef tallow.

EXPERIMENTAL

Materials. [U-^{14}C]sucrose, specific activity 25 μCi/mg, was obtained from the Radiochemical Centre, Amersham, Bucks. Grade 1 tallow, iodine value 44 ± 1, was obtained from Oleofina, Brussels, Belgium. Analysis of the fatty acid radicals in the tallow showed it to contain 27% palmitic acid, 22·5% stearic acid, 41·2% oleic acid and 3·1% myristic acid. Insta-gel®, Carbosorb®, Permafluor®, Cumbustucones® and Soluene 350® were obtained from the Packard Instrument Company, Caversham, Berks. Pancreatin, from pig pancreas, was obtained from B.D.H. Ltd., Poole, Dorset. Sucrose tallowate, containing 85% mono- and diesters and 50 ppm ethyl acetate was prepared, as described in UK Patent Nos 1399053 and 1500341, by a process that does not require the use of dimethylformamide. All other reagents were of Analar grade and were obtained from Fisons Scientific Apparatus Ltd., Crawley, Sussex.

Preparation of [U-^{14}C]sucrose tallowate. [U-^{14}C]-sucrose (2 g; 4 mCi), potassium carbonate (0·6 g) and tallow (4·42 g) were reacted together at 125°C under a blanket of dried nitrogen, using the procedure described in UK Patent No. 1399053. The product (7·0 g) was suspended in demineralized water (30 ml), an aqueous solution of calcium chloride (0·7 g) was added and the solids were coagulated by heating the suspension to 65°C. The solids were collected and freeze-dried and the glyceride fraction was removed by trituration with dry ethyl acetate (3 × 25 ml). The residue was dried *in vacuo* and extracted with propan-2-ol (3 × 25 ml). Evaporation of the combined extracts yielded a mixture of [^{14}C]sucrose esters (1·05 g). In order to remove sucrose, monoglyceride and calcium soaps completely, this product was further purified as follows: The esters were dissolved in a minimum volume of dichloromethane and the solution was applied to a column (400 × 25 mm) of silica gel (80 g) in ether–*n*-butanone–water (25:25:1, by vol.). The column was eluted with the solvent mixture and fractions (25 ml) were collected. The eluant was changed to water-saturated *n*-butanone at fraction 20 and a further 12 fractions were collected. Fractions 6–14 and 15–26, containing sucrose diesters (120 mg) and sucrose monoesters (262 mg) respectively, were combined and concentrated. The residues were dissolved in chloroform, and the solutions were combined and concentrated to give a product (316 mg) of [^{14}C]sucrose tallowate, specific activity 0·7 μCi/mg.

Balance study. Adult male rats of the Charles River CD strain (body-weight range 220–260 g) were given,

either by oral intubation or by ip injection, a solution of [^{14}C]sucrose tallowate in propylene glycol in a single dose of 5, 50 or 100 mg/kg body weight. Animals were housed in individual all-glass metabolism cages ('Metabowl', Jencons Scientific Limited, Hemel Hempstead) and allowed free access to food and water. Air was drawn through the system at a constant rate of 300 ml/min and then through two Dreschel bottles in series each containing 2 M-NaOH (100 ml). Aliquots were taken at intervals during the first 6 hr and thereafter at 24-hr intervals for the analysis of $^{14}CO_2$. Urine and faeces were collected separately and analysed at intervals of 24 hr. Similar studies were conducted in male rats given an aqueous solution of [U-^{14}]sucrose, either orally or by ip injection, in a single dose of 2·5 and 25 mg/kg body weight respectively.

Accumulation studies. A group of nine adult male rats were intubated daily for 21 days with [^{14}C]sucrose tallowate (100 mg/kg body weight). Each animal received approximately 0·1 μCi radioactivity/day. Groups of three animals were killed on days 7, 14 and 21, and liver and adipose tissue were removed. An additional group of three animals was treated for 14 days with [^{14}C]sucrose tallowate and killed on day 21.

Human studies. Sucrose tallowate (1 g) was administered in a mixture of butter and cream cheese to three volunteers weighing 69, 61·7 and 65·3 kg respectively. The 24-hr urines were collected and analysed for sucrose using the procedure described below. Subsequently, each volunteer was given a solution of sucrose (10 g) in water (100 ml), and the urine was collected and similarly analysed.

In vitro *studies.* A 10% (w/v) homogenate of rat liver was prepared in Krebs-Ringer phosphate buffer (pH 7·4) containing 0·1% glucose and the suspension was centrifuged at 3000 rev./min for 10 min. Portions (3 ml) of the supernatant were incubated at 37°C, with shaking, with [^{14}C]sucrose tallowate (250 μg/ml). Aliquots (0·5 ml) were removed at 0, 1, 2, 3, 4 and 5 hr and assayed for sucrose tallowate and sucrose by chromatography on thin-layer plates of silica gel 60 F_{254} (Merck AG, Darmstadt). Chromatograms were developed in *n*-butanol–acetone–water (4:5:1, by vol.) and scanned with a Packard radiochromatogram scanner (Model 7201). Areas of the support corresponding to radioactivity were removed and assayed by liquid scintillation spectrometry. [^{14}C]Sucrose tallowate (250 μg/ml), 70 μmol Tris buffered at pH 8·1 and pancreatin (100 μg) were incubated at 37°C and aliquots (250 μl) were removed at 0, 1, 2, 3, 4, 5, 6 and 24 hr, and assayed for sucrose as described.

Radiochemical analyses. Urine (1 ml) and extracts of expired air were added to Instagel (15 ml). Faeces were homogenized in water (1:3, w/v) using a Silverston top-drive homogenizer and samples (100–150 mg) were combusted using a Packard 306 sample oxidizer. The liberated $^{14}CO_2$ was absorbed in Carbosorb (7 ml) and the solution was added to Permafluor V (14 ml).

Portions of liver were analysed by combustion, while adipose tissue (0·5 g) was solubilized in Soluene 350 (3 ml) and diluted to 5 ml with propan-2-ol. Aliquots (2 ml) were added to Instagel (15 ml). The radioactivity of all samples was determined using a Packard 3330 Tricarb liquid scintillation spectrometer. Counting efficiency was determined by the twin channels ratio technique or by the use of internal standards.

Determination of sucrose. Urine (20 ml) was freeze-dried and the residue was dissolved in pyridine (5 ml). Trimethylchlorosilane (1 ml) and *N,O*-bistrimethylsilylacetamide (1 ml) were added and the mixture was heated at 70°C for 30 min. An aliquot (2 μl) was examined by gas chromatography on a Pye GCV gas chromatograph, equipped with a flame-ionization detector, using a 1·5 m × 0·4 mm ID glass column packed with 3% SE52 on Chromosorb WHP (80–100 mesh). The carrier gas was nitrogen (flow-rate 50 ml/min), the column temperature 250° and the detector temperature 300°C. The limit of detection was equivalent to 0·5 ppm in the urine.

RESULTS

Rats given a single oral dose of [^{14}C]sucrose tallowate excreted an average of 5% of the radioactivity in the urine and 11% in the faeces within 96 hr (Table 1). Most (61·5%) of the radioactivity was recovered from the expired air, the rate of elimination being independent of dose. In similar experiments using [^{14}C]sucrose, 4 and 66% of the activity was recovered from the urine and expired air within 96 hr.

When [^{14}C]sucrose tallowate was administered by ip injection, 61% of the activity was excreted in the

Table 1. *The excretion of radioactivity by male rats given either sucrose tallowate or sucrose orally or by ip injection*

Test compound	Route	Dose (mg/kg)	Sampling period (hr)	Radioactivity (%) excreted in		
				Expired air	Urine	Faeces
Sucrose tallowate	Oral	5	96	58·2, 62·4	5·1, 5·2	9·9, 13·8
		50		53·9, 55·7, 59·5	4·2, 4·4, 3·9	6·1, 8·4, 8·0
		100		49·9, 55·9	4·9, 4·1	19·2, 14·5
	Intraperitoneal	5	96	18·0, 18·7	69·5, 67·6	2·7, 1·9
		50		16·8, 19·8	50·9, 62·1	1·6, 1·9
		100		19·3, 19·6	57·1, 58·4	0·6, 1·2
Sucrose	Oral	2·5	48	70·3, 67·2	3·6, 3·7	1·4, 1·3
		25		62·0, 65·8	5·4, 3·9	2·3, 1·4
	Intraperitoneal	2·5	48	2·1	90·3	—*
		25		0·0, 0·5	79·1, 74·2	—*

*Not analysed.

Table 2. *The retention of radioactivity in the adipose tissue and liver of rats dosed daily during a period of 21 days with [^{14}C]sucrose tallowate (100 mg/kg/body weight)*

Tissue	Percentage of dose/g tissue at day 7	14	21	21*
Fat	0·21	0·08	0·05	0·09
	0·19	0·18	0·04	0·11
	0·19	0·06	0·05	0·09
Liver	0·07	0·03	0·02	0·01
	0·06	0·03	0·01	0·01
	0·05	0·02	0·01	0·01

*14-day treatment followed by 7-day recovery period.

urine, while an additional 19% was recovered from the expired air in 96 hr. When [^{14}C]sucrose was administered by the same route, approximately 82% of the radioactivity was excreted in the urine within 24 hr.

Chromatography of 24 hr urine samples obtained after ip administration of [^{14}C]sucrose tallowate indicated the presence of only a single radioactive component, which corresponded to sucrose. Examination of urine obtained after oral administration of the ester failed to reveal the presence of either sucrose or unhydrolysed esters.

No evidence was obtained to indicate any progressive accumulation of sucrose tallowate in either the liver or adipose tissue of rats following the daily oral administration of sucrose esters for 21 days (Table 2). In comparable studies in which [^{14}C]sucrose was administered daily for 7 days, radiochemical analysis of adipose tissue indicated that approximately 0·13% of the label had been retained. It is considered that the radioactivity in adipose tissue following the administration of sucrose tallowate represents the incorporation into lipids of labelled precursors derived from glucose and fructose.

Analysis of the 24-hr human urines following the administration of sucrose tallowate indicated the presence of 6, 12 and 13 ppm sucrose respectively. The clearance of sucrose was subsequently investigated in the three subjects each given 10 g sucrose. Values of 7, 15 and 24 ppm were obtained for the concentration of sucrose in urine.

When sucrose tallowate was incubated with rat liver homogenates, approximately 20% of the available sucrose was liberated in 6 hr. No hydrolysis occurred when the esters were incubated with pancreatic lipase under the conditions described above.

DISCUSSION

The data indicate that the sucrose esters prepared from beef tallow are almost completely absorbed after oral administration to rats at dosages equivalent to forty times the recommended acceptable daily intake. The low levels of radioactivity in the rat urine and of sucrose in the urine of human volunteers provide presumptive evidence that the esters are hydrolysed to sucrose and the corresponding fatty acids before absorption.

The mechanism of hydrolysis is uncertain for while it may be anticipated that the esters will be unstable under the acidic conditions of the stomach, no evidence was obtained for the liberation of sucrose when the esters were incubated *in vitro* with a pancreatic extract. This is in accord with the observations of Berry & Turner (1960), who reported that hydrolysis required the presence of bile acids. The similarity in the distribution of radioactivity following the administration of either sucrose tallowate or sucrose is additional evidence that little, if any, of the ester is absorbed intact.

The rapid elimination of ^{14}C when the esters were adminstered parenterally and the relative ease with which they are hydrolysed by homogenates of rat liver further suggest that any intact ester absorbed is unlikely to accumulate in the tissues. This was confirmed by the analysis of adipose tissue and liver of rats after the repeated administration of sucrose tallowate, the levels of radioactivity demonstrated being essentially similar to those obtained with [^{14}C]sucrose.

Because of the apparent ease with which the esters are hydrolysed under physiological conditions to sucrose and the corresponding fatty acids, the use of these materials as additives to human foods would not appear to present a significant toxicological hazard.

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LONG-TERM TOXICITY STUDY OF QUILLAIA EXTRACT IN MICE

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Abstract—Groups of 48 male and 48 female mice were fed quillaia extract in the diet at levels of 0 (control), 0·1, 0·5 or 1·5% for 84 wk. The material had no adverse effect on the death rate or the incidence of histopathological findings, including tumours. However, there was a lower rate of body-weight gain at the 1·5% dietary level, and there were isolated statistically significant differences between the treated and control animals, mainly at the 1·5% dietary level, in the haematological examinations and in some absolute and relative organ weights of both sexes. It is concluded that, in mice, quillaia extract fed at levels up to 1·5% in the diet (approximately 2·2 g/kg/day) did not exert a carcinogenic effect. The no-untoward-effect level from this study is considered to be 0·5% in the diet, giving an intake of approximately 0·7 g quillaia extract/kg/day.

INTRODUCTION

Saponins are the glycosides of a number of related steroids or triterpenes. The most commonly found carbohydrate moeity is glucose, although galactose, arabinose, xylose and rhamnose have been identified in the wide range of saponins examined (Jackson & Shaw, 1959; Marker, Wagner, Ulshafer, Wittbecker, Goldsmith & Ruof, 1947). The saponins from quillaia species are of the triterpene glycoside type and in this material two major and two minor components have been identified (Cheeke, 1971; Coulson, 1958).

The amphipathic properties of saponins have led to the use of quillaia extract as an emulsifier and foaming agent, particularly in soft drinks. The material is also widely used in toiletry preparations and pharmaceuticals. Quillaia extract is permitted in the UK in soft drinks, at not more than 200 ppm (w/w) under The Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486). This followed recommendations from the Food Additives and Contaminants Committee (1970 & 1972) that the future inclusion of extracts prepared from quillaia of British Pharmacopoeia standard was provisionally acceptable for restricted food use subject to a requirement for further data from long-term studies.

The toxicological data on quillaia extract were reviewed by Gaunt, Grasso & Gangolli (1974), who found a no-untoward-effect level for quillaia of 0·6% of the diet (equivalent to an intake of approximately 400 mg/kg/day) in a short-term study in rats. The findings at the 2 and 4% dietary levels were confined to lower liver weights and higher stomach weights.

The present paper gives the results of a long-term study of the effects of quillaia extract in mice, carried out as part of a safety evaluation programme, which included short-term (Gaunt *et al.* 1974) and long-term work on this material in the rat.

EXPERIMENTAL

Materials. The quillaia extract used in this study was supplied by Food Industries Ltd., Bromborough Port, Wirral, Merseyside. It was a spray-dried aqueous extract of quillaia bark, prepared in such a manner that 100 parts by weight of bark yielded approximately 15 parts of extract before drying. The extract was stated to contain less than 10% moisture and less than 10% ash (at 550°C). It conformed to the requirements of The Emulsifiers and Stabilizers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486).

Animals and diet. Mice of the TO strain obtained from a specified-pathogen-free breeding colony (A. Tuck & Son, Rayleigh, Essex) were used. They were housed at 21 ± 1°C in a relative humidity of 50–60%, and were given water and ground Oxoid pasteurized breeding diet supplemented with vitamin K, *ad lib.*

Experimental design and conduct. Groups of 48 male and 48 female mice were fed diets containing 0, 0·1, 0·5 or 1·5% quillaia extract for 84 wk. The males were caged singly and the females in groups of four. The condition and behaviour of the animals were observed frequently and sixteen of the individually caged mice were weighed at intervals. Any mouse showing signs of ill-health was closely observed and was killed if its condition deteriorated. An autopsy was conducted on all animals unless this was precluded by advanced autolysis.

At the end of the study the surviving animals were killed by exsanguination from the aorta under barbiturate anaesthesia. At autopsy, all macroscopic abnormalities were noted and the brain, heart, liver, kidneys, spleen, stomach, small intestine, caecum and testes were weighed. Samples of these organs, together with salivary gland, thyroid, adrenal glands, lymph nodes, aorta, pancreas, pituitary, prostate, seminal vesicles, ovaries, uterus, urinary bladder, lungs, colon, rectum, spinal cord, skeletal muscle, eye, Harderian gland and other tissues that appeared abnormal were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

Blood samples were collected from a caudal vein of each of ten male and ten female mice from the

Table 1. *Cumulative mortality record of mice fed diets containing 0–1·5% quillaia extract for 84 wk*

	Cumulative mortality in							
	Males given dietary levels (%) of				Females given dietary levels (%) of			
Wk	0	0·1	0·5	1·5	0	0·1	0·5	1·5
26	0	1	0	0	0	0	1	0
52	4	3	3	1	0	4	3	2
64	10	6	6	4	2	8	5	4
72	12	10	9	5	4	10	6	9
80	16	18	12	9	9	11	10	12

Figures represent the number of mice dead or killed *in extremis* from groups of 48. There was no significant positive trend in cumulative mortality due to treatment ($P > 0·05$) using the logrank conditionally expected numbers method (Peto & Pike, 1973).

control group and those on the two higher dietary levels at wk 26 and 54 and from all surviving mice at wk 84. The blood samples were examined for haemoglobin concentration and packed cell volume, and counts were made of reticulocytes and total erythrocytes and leucocytes.

RESULTS

The ingestion of quillaia extract had no effect on the condition or behaviour of the animals. There were deaths in all groups during the course of this study, but there was no relationship between the number of deaths at any time and the dietary level of quillaia extract (Table 1). The body weights of all treated males (Table 2) were similar to those of the controls at each weighing, but overall there was a slightly lower gain in weight in the males given 1·5% quillaia extract. The difference between this group and the controls was statistically significant at autopsy.

The weights of the testes were lower ($p < 0·05$) in the mice fed 0·5 or 1·5% quillaia extract, but the differences from the control value were not statistically significant when the weights were expressed relative to body weight (Table 3). Other isolated organ weights differed significantly from those of the controls, but in each case the differences occurred in one sex only and were not apparent in both the absolute and relative figures. However, they all occurred in the mice that received the highest dietary level. The significant absolute values were reduced liver and kidney weights in males and a high small-intestine weight in females.

The erythrocyte counts were lower in some of the animals given 0·5 or 1·5% quillaia extract in the diet than in their controls, but these findings were not dose-related and were not accompanied by higher reticulocyte counts (Table 4). Also at wk 84 there was a higher packed cell volume in males fed the 1·5% dietary level, while this value was lower in the females given the 0·5% level. There were no significant differences from the corresponding control values in the other haematological measurements.

The histopathological findings are summarized in Table 5. There were no significant differences between treated and control mice in the incidence of the lesions and there was a tendency for fewer lesions to be seen in the group on the highest level of quillaia. The tumours found are listed in Table 6. A number of malignant tumours were detected, but these were mainly in the control groups. No malignant tumours were found in any of the treated female mice and none occurred more frequently in the treated than in the control males. The total incidence of malignant tumours in the three groups of treated mice was less than that in the controls. The most frequently found benign tumours were those of the lung and Harderian gland, but in no instance was there any evidence of a dose-related effect on tumour incidence or of a statistically significant incidence in the treated animals compared to the controls. In addition the total incidence of these benign tumours was similar in all groups.

Table 2. *Mean body weight of male mice fed diets containing 0–1·5% quillaia extract for 84 wk*

	Body weight (g) at wk								
Dietary level (%)	0†	1	4	10	14	28	40	57	84
0	30	32	37	41	45	52	56	59	58
0.1	31	32	36	40	44	52	58	60	58
0·5	30	31	35	41	45	52	59	61	59
1·5	32	32	35	38	42	50	56	58	56

† Value on first day of feeding.
The figures are means for 16 mice and those for treated animals did not differ significantly ($P > 0·05$ by Student's t test) from those of the controls.

Table 3. *Relative organ weights of mice fed diets containing 0–1·5% quillaia extract for 84 wk*

Organ	Dietary level (%)...	Relative organ weight (g/100 g body weight) 0	0·1	0·5	1·5
		Males			
Brain		0·85	0·88	0·91	0·96*
Heart		0·48	0·52	0·50	0·51
Liver		3·67	3·75	3·85	3·75
Spleen		0·26	0·32	0·38	0·25
Kidney		1·27	1·31	1·31	1·28
Stomach		0·71	0·75	0·77	0·82*
Small intestine		4·12	4·31	4·19	4·37
Caecum		0·38	0·40	0·42	0·41
Testes		0·57	0·55	0·53	0·57
Terminal body weight (g)...		53	51	51	47*
		Females			
Brain		1·00	1·04	1·01	1·03
Heart		0·39	0·39	0·40	0·42
Liver		4·07	3·95	4·14	4·14
Spleen		0·41	0·31	0·46	0·41
Kidney		1·03	1·03	1·04	1·04
Stomach		0·93	0·91	0·85	0·97
Small intestine		3·80	3·95	3·92	4·56
Caecum		0·46	0·49	0·44	0·47
Terminal body weight (g)...		46	43	45	43

Figures are means for groups of 31 males or 37 females and those marked with an asterisk differ significantly (Student's t test) from those of the controls: $*P < 0.05$.

DISCUSSION

It is notoriously difficult to detect marginal differences in rates of body-weight gain in mice in view of their small size and relatively small weight gain. Nevertheless the weight gain of the small sample of mice fed 1·5% quillaia extract was slightly less (15%) than that of the controls during the study. When all the surviving mice were weighed during the post-mortem examination, the same group showed a significantly lower body weight than the controls. On this evidence, some slight effect on the body weight

Table 4. *Results of haematological examinations in mice fed diets containing 0–1·5% quillaia extract for 26 or 84 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of REC)	WBC (10^3/mm^3)
			Wk 26			
Male						
0	10	17·2	53	9·98	1·7	15·1
0·5	10	15·4	49	8·30*	1·9	14·5
1·5	10	16·9	54	8·82**	1·8	16·5
Female						
0	10	16·4	53	9·53	1·0	12·2
0·5	10	16·6	53	9·06	1·4	14·7
1·5	10	16·0	51	8·55**	1·4	14·8
			Wk 84			
Male						
0	31	14·6	47	7·68	2·1	11·4
0·1	28	14·5	45	7·07	3·1	12·1
0·5	33	14·3	45	6·71**	2·2	10·5
1·5	37	15·6	50*	7·78	1·4	12·6
Female						
0	37	15·1	49	8·50	2·1	10·2
0·1	35	14·8	48	8·01	2·1	9·4
0·5	33	14·3	46*	8·09	1·8	11·5
1·5	34	14·6	47	8·07	2·6	10·2

Hb = Haemoglobin. PCV = Packed cell volume. RBC = Red blood cells. Retics = Reticulocytes. WBC = White blood cells

Figures are means for the numbers of mice shown and those marked with asterisks differ significantly (Student's t test) from those of the controls: $*P < 0.05$; $**P < 0.01$.

Table 5. *Incidence of histopathological findings (excluding tumours) found in mice fed diets containing 0–1·5% quillaia extract for 84 wk*

Tissue and histological finding	No. of mice affected							
	Males fed dietary level (%) of				Females fed dietary level (%) of			
	0	0·1	0·5	1·5	0	0·1	0·5	1·5
No. of mice examined...	45	42	41	43	44	43	43	46
Lung								
Chronic inflammatory infiltration	1	0	0	1	0	0	0	0
Liver								
Focal areas of vacuolation	12	12	5	14	23	16	22	14
Severe fatty change	5	3	9	3	7	9	5	1
Necrotic foci	11	5	5	1	3	2	0	0
Amyloid	2	3	0	0	0	0	0	0
Kidney								
Degenerative changes	11	13	9	9	13	5	7	3
Bladder								
Foci of chronic inflammation	4	3	1	2	2	0	1	0
Heart								
Chronic degenerative changes	2	1	2	0	0	0	0	0
Gastro-intestinal tract								
Chronic inflamation and degenerative changes	0	2	1	3	1	0	0	0
Testes								
Atrophy	3	1	1	1	—	—	—	—
Foci of chronic inflammation in seminal vesicles	3	3	1	0	—	—	—	—
Ovary								
Follicular cyst	—	—	—	—	5	6	10	8
Non-specific								
Chronic subcutaneous abscess	1	0	1	4	0	0	0	1
Calcified areas in tissues	5	3	2	1	3	1	0	0

The figures for the treated groups were not significantly greater ($P > 0·05$ by the chi-square test) than those of the controls.

at this highest treatment level cannot be excluded. This material has been reported to reduce the rate of body-weight gain when fed to rats and chickens (Coulson & Evans, 1960; Newman, Kummerow & Scott, 1958; Peterson, 1950). In the previous study in rats (Gaunt *et al.* 1974), a transitory effect on the rate of body-weight gain was observed but was attributed to the unpalatability of the diet rather than to a toxic effect. It was further suggested that the effect might have been due to local irritation of the gastro-intestinal tract by the high concentrations (2 or 4%). Food intake was not measured in the present study, but the similarity of the dietary levels in this and the rat study suggests the possibility of a similar cause in both species, although, as in the rats, there was no evidence in the mice of any direct effect on the gastro-intestinal tract. Gaunt *et al.* (1974) suggested that any direct effects on the alimentary tract are likely to be concentration- rather than dose-related and hence of little relevance to the assessment of risk in man.

Following treatment at the highest level, there were isolated statistically significant changes in the weights of the liver and kidneys in males and in the small intestine in females, and there were also increased relative brain and stomach weights in the male mice. Lower liver and higher gastro-intestinal weights were reported in the short-term rat study (Gaunt *et al.* 1974) and therefore it is possible that they represent a toxic effect although, in the present study, no pathological changes were seen on histological examination in the gastro-intestinal tract.

There were slightly lower testicular weights in the mice given 0·5 or 1·5% quillaia extract in the diet compared with the controls. However, this effect was not apparent when the weights were expressed relative to body weight and there was no evidence of pathological changes on histological examination. Thus these observations are unlikely to be of toxicological significance.

The absence of any malignant tumours in the treated female mice and the low incidence in the males given quillaia extract indicates a lack of any carcinogenic effect. The similarity of the incidences of benign tumours in all the groups is also indicative of a lack of carcinogenic effect. Consideration of the individual tumours does not suggest any specific effect on any organ. The tumours found in treated mice without comparable findings in the controls were of low incidence generally, only one tumour being found. This is suggestive of a spontaneous occurrence. This is supported by the general similarity of the tumour incidence in the present experiment to that expected in old mice (Cloudman, 1956; Tucker & Baker, 1967). The occasional sebaceous adenoma, for example, may represent a spontaneous occurrence, since this tumour has been reported by Murphy (1966) to occur spontaneously in mice.

Table 6. *Incidence of tumours in mice fed diets containing 0–1·5% quillaia extract for 84 wk*

	No. of mice affected							
	Males fed dietary levels (%) of				Females fed dietary levels (%) of			
	0	0·1	0·5	1·5	0	0·1	0·5	1·5
Tissue and tumour — No. of mice examined...	45	42	41	43	44	43	43	46
Liver								
Malignant hepatoma	5	1	2	1	0	0	0	0
Haemangioma	1	0	0	0	0	0	0	0
Haemangiosarcoma	1	0	0	0	0	0	0	0
Hyperplastic nodules	8	4	3	6	1	2	1	0
Lung								
Papillary adenoma	7	3	7	12	9	5	4	5
Pituitary								
Adenoma	0	0	0	0	1	1	0	0
Harderian gland								
Adenoma	2	1	1	0	3	0	0	1
Stomach								
Fibrosarcoma	0	0	0	0	1	0	0	0
Uterus								
Fibromyoma	—	—	—	—	1	1	1	0
Adenocarcinoma	—	—	—	—	1	0	0	0
Ovary								
Granulosa cell tumour	—	—	—	—	0	1	0	0
Testis								
Interstitial cell tumour	0	0	1	0	—	—	—	—
Breast								
Adenocarcinoma	—	—	—	—	1	0	0	0
Reticulo-endothelial system								
Sarcoma in lymph node	1	0	0	0	0	0	0	0
Skin and subcutaneous tissue								
Squamous papilloma	0	0	1	0	0	0	0	0
Subcutaneous fibroma	1	0	0	0	0	0	0	0
Subcutaneous fibrosarcoma	0	0	0	0	1	0	0	0
Sebaceous gland adenoma	0	0	0	0	0	0	0	1
Sebaceous gland adenocarcinoma	0	0	0	0	1	0	0	0

The figures represent the incidence of tumours in the numbers of mice shown; those for the treated groups were not significantly greater ($P > 0{\cdot}05$ by the chi-square test) than those of the controls.

On the basis of the results of this study in mice, there is no evidence that quillaia extract has a carcinogenic effect when given at dietary levels up to 1·5%, a level providing an intake of approximately 2·2 g/kg/day. However, the slightly lower rate of body-weight gain in the mice on the highest dietary level and some organ-weight changes, albeit of doubtful significance, indicate a no-untoward-effect level for quillaia extract of 0·5% of the diet in this study, providing an intake of approximately 700 mg/kg/day.

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CONTAMINATION OF BEER WITH TRACE QUANTITIES OF *N*-NITROSODIMETHYLAMINE

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Abstract—Chemiluminescence detection was used to analyse 158 samples of commercially available draft, bottled and canned beers of different types for contamination with volatile nitrosamines. Of these samples, 111 (70%) were found to contain *N*-nitrosodimethylamine, the mean concentration being 2·7 ppb (μg/kg) and the maximum level 68 ppb. *N*-Nitrosodiethylamine, the only other volatile nitrosamine found, was detectable in only two samples (at 0·5 and 3·0 ppb).

INTRODUCTION

Among more than 2500 samples of food analysed for volatile nitrosamines in an ongoing screening of food products from the German market, a series of beer specimens was investigated. Low concentrations of *N*-nitrosodimethylamine (NDMA) were consistently found. To obtain more detailed information about the occurrence of NDMA in beer, a greater variety of bottled, canned and draft beers, widespread in origin, was examined.

EXPERIMENTAL

Samples of bottled or canned beers were obtained in retail shops and supermarkets in Heidelberg between October 1977 and April 1978. Draft beer was obtained directly from the tap and transferred immediately to glass vessels.

The samples (5–20 ml) were analysed for volatile *N*-nitroso compounds according to a modification (by B. Spiegelhalder, G. Eisenbrand and R. Preussmann, unpublished data 1978) of the procedure of Fine, Rufeh, Lieb & Rounbehler (1975). After addition of an internal standard (200 ng *N*-nitrosodipropylamine), the flask containing the sample, 20 ml paraffin oil and 4 ml aqueous 0·1 M-NaOH was attached to a distillation bridge connected to a 100-ml long-necked round-bottomed receiving flask, cooled at −70°C with acetone/solid CO_2. After evacuation with an oil pump, the connection to the pump was shut and the sample was slowly heated. During heating, vacuum was applied periodically for a few seconds, so that distillation was performed in a practically closed system. The distillation was terminated when the temperature reached 100°C (after 40–50 min). The distillate was then either extracted three times with three equal volumes of CH_2Cl_2 at pH 1 (H_2SO_4) or subjected to liquid–liquid partition on kieselguhr (Extrelut®; Merck AG, Darmstadt) with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and concentrated in a Kuderna-Danish evaporator; 2 ml *n*-hexane was added and a final volume of 1 ml was attained by a stream of nitrogen. Blank values using the same amounts of reagents were prepared in the same way.

Volatile *N*-nitroso compounds were determined by gas chromatography with a chemiluminescence detector (TEA detector, Thermo Electron Corp., Waltham, MA, USA). Samples with a nitrosamine content above 3 ppb were confirmed by high-resolution mass spectrometry (resolution 12,000; peak-matching technique).

The lower detection limit of this screening method was 0·1 ppb.

RESULTS AND DISCUSSION

Of the 158 samples analysed for volatile nitrosamines, 111 (70%) were found to be contaminated with NDMA (Table 1). The mean of the NDMA concentrations in all samples was 2·7 ppb and the highest value obtained was 68 ppb. Only 47 samples (30%) were negative or contained less than 0·5 ppb, while 54 samples (34%) contained between 0·5 and 1·4 ppb, 41 samples (26%) ranged from 1·5 to 4·9 ppb, and 16 (10%) contained more than 5 ppb with a surprising maximum value of 68 ppb. Unpublished results of R. Stephany and P. Schuller (personal communication 1978) agree with our values and the two sets of data may therefore be assumed to reflect very well the current NDMA contamination of beer.

N-Nitrosodiethylamine was the only other volatile nitrosamine found, but it was detectable only in two samples, at concentrations of 3·0 and 0·5 ppb, respectively.

Classification according to the type of beer shows that Pilsen lager, pale Export lager and especially top-fermented pale ales had relatively low levels of NDMA contamination. The higher values were found either in beers with a very high original gravity like strong beer or in beers for which the malt production involved a special treatment, like high kilning and curing temperatures.

In the group of special beers, the samples with the highest levels of NDMA (4–68 ppb) were those of the so-called 'Rauchbier', which is made from smoked malt to give a smoky taste.

The proportion of total beer sales accounted for by each of the most important types (Brauwelt-Report, 1978) is given in Table 2 together with the

Table 1. N-*Nitrosodimethylamine (NDMA) in different types of beer

Type of beer	No. of samples	No. of samples with NDMA concn (ppb) of < 0·5 (negative)	0·5–1·4	1·5–4·9	5·0 or more	NDMA concn (ppb) Mean	Maximum
Pilsen lager	49	21	17	9	2	1·2	6·5
Pale Export lager	27	8	9	9	1	1·2	6·5
Top fermented pale ales	8	7	1	—	—	0·2	0·5
Pale strong lager beer	23	5	6	10	2	2·0	5·0
Top fermented dark ales	13	2	4	6	1	2·4	9·5
Dark lager and dark strong lager beers	8	—	4	—	4	11·2	47
Alcohol-free lager beers	6	2	2	2	—	1·6	4·0
Special types of beer*	24	2	11	5	6	6·8	68
All types	158	47	54	41	16	2·7	68

* 'Rauchbier' and beers that are not produced in West Germany, for example.

Table 2. *Current sales of different beers in West Germany (1977), as a percentage of total beer sales, compared with their mean concentration of NDMA*

Type of beer	Share of total market (%)	Mean NDMA concn (ppb)*
Pilsen lager	40–45	1·2
Pale lager and pale Export lager	40–45	1·2
Top fermented pale and dark ales	14	1·6
Pale and dark strong lager beers	1	4·5

* Overall (adjusted) mean 1·3 ppb.

corresponding mean concentrations of NDMA. The total amount of beer produced in West Germany in 1977 was 91,000,000 hectolitres. The consumption per head was 148 litres/year, or 565 g beer/day for males and 255 g/day for females (Deutsche Gesellschaft für Ernährung e.V., 1976).

In view of the high daily consumption of beer, even low concentrations of carcinogenic NDMA must be considered an important contribution to human exposure to preformed nitrosamines in food. On the basis of an adjusted mean value, related to the actual percentage of sales of the different types of beer, a mean intake of 0·7 μg NDMA/day for males and 0·3 μg/day for females can be calculated. In the age groups with the highest levels of consumption (36–50 years for males and 19–35 years for females), the mean intakes are 1·2 and 0·5 μg/day, respectively. These values may be lower or higher depending on the type of beer individually preferred.

The dimethylamine (DMA) content of beer ranges between 200 and 300 μg/kg, and is higher in dark than in pale beers (Hrdlicka, Dyr & Kubickova, 1964). One possible source of DMA in beer is hordenine (*N,N*-dimethyl-*p*-hydroxyphenylethylamine) which is formed in the barley kernels during malting (Drews, Just & Drews, 1958). NDMA could be formed from DMA during the brewing process, if traces of nitrosating agents were available at that stage; nitrite could be formed from nitrate during processing or nitrogen oxides might be present. A further source of nitrite might be the use of anticorrosion cleaning fluids and/or inorganic filter material (kieselguhr). The possible importance of DMA in the malt as a precursor for NDMA in beer is underlined by the evidence that DMA concentrations in dark lager, dark strong lager and dark ale are relatively high and correspond with higher NDMA values (Table 1).

Studies designed to elucidate the origin and formation of NDMA in beer are in progress. First results indicate that malt is the main source of contamination.

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LONG-TERM TOXICITY AND CARCINOGENICITY STUDIES OF THE BREAD IMPROVER POTASSIUM BROMATE
1. STUDIES IN RATS*

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Abstract—Wistar rats were fed for 104 wk on five bread-based diets in which the bread was prepared from untreated flour or from flour treated with 50 or 75 ppm potassium bromate or with one of two mixtures of potassium bromate with other commonly-used additives. Appearance, behaviour and health were similar in test and control groups. The death rate was lower in the test groups than in the controls in the females, and the males of the high-dose group experienced significantly fewer deaths than the other groups taken together. No evidence of carcinogenicity and no definite evidence of chronic toxicity was attributable to the compounds under test. There was no evidence of retention or accumulation of covalent bromine in the adipose tissues of the rats.

INTRODUCTION

Potassium bromate has been used in the UK for some 50 yr to oxidize certain flour constituents and thus to produce well-risen loaves with acceptable keeping properties. Ascorbic acid (oxidant), benzoyl peroxide (bleach) and/or chlorine dioxide (oxidant, mild bleach) may additionally be used. Potassium bromate is quantitatively converted to the bromide during dough-mixing if the concentration in the flour does not exceed 75 ppm, above which level residues of unchanged bromate remain in the bread (Thewlis, 1974). Earlier work on the toxicology of potassium bromate has been reviewed by the Joint FAO/WHO Expert Committee on Food Additives (1964). Long-term studies of toxicity and carcinogenicity involving bread-based diets were lacking, and are the subject of this and a subsequent paper (Ginocchio, Waite, Hardy, Fisher, Hutchinson & Berry, 1979).

EXPERIMENTAL

Diets. All additives were in forms normally used commercially. Potassium bromate (*Food Chemicals Codex* 1972, p. 643) was supplied by Akzo Chemie U.K. Ltd., London, as a white powder containing 10% crystalline potassium bromate together with inorganic diluents. Benzoyl peroxide (*Food Chemicals Codex*, 1972) was added at the mill as a 16% mixture with inorganic diluents supplied by Engineering and Chemical Supplies (Epsom & Gloucester) Ltd., Gloucester. Chlorine dioxide, a gaseous improver, was generated at the mill in plant supplied by Wallace & Tiernan, Tonbridge, Kent, and may have contained about 20% of chlorine. Ascorbic acid was the BP grade (Roche Products Ltd., London).

The grist used at each monthly milling (Heygates Ltd., Tring, Herts.) was identical for all three flours. Stringent supervision and analytical control was maintained over the production, treatment and composition of the flours. Flour was made into bread using the Chorleywood Bread Process (Recipe (parts by weight): flour 100, yeast 2·1, salt 1·8, fat 0·7, additives as specified in Table 1, and water according to the water absorption of the flour). The types of flour and bread produced are shown in Table 1.

Bread was crumbed and dried at 49°C in a stream of filtered air for incorporation in the following diet (% by weight): breadcrumbs (moisture content approximately 12%) 79, casein 8, minerals (Jones & Foster, 1942) 4, wheat bran, autoclaved 3, corn oil 2·9, arachis oil 1·8, L-lysine hydrochloride 0·3, and a vitamin mixture 1. The vitamin mixture comprised (mg or IU/g): thiamine 0·5, riboflavine 1·0, pyridoxine 0·5, nicotinic acid 0·5, vitamin B_{12} 0·0005, folic acid 0·5, calcium pantothenate 2·5, choline chloride 100·0, biotin 0·05, *p*-aminobenzoic acid 15·0, α-tocopherol 7·5, menaphthone 1·5, vitamin A 500 IU, vitamin D 60 IU, and icing sugar to 1 g.

This diet contained about 15% protein, 6% total fat and 63% available carbohydrate (moisture content 10–12%); energy content was 15·5 kJ/g. The five diets differed only in respect of the additives specified in Table 1.

*Full experimental details and an extended discussion of the results are available on loan from FMBRA.

Table 1. *The types of bread used in trials of potassium bromate*

Bread/ diet code	Flour code	Additives (ppm based on flour weight) Bromate	Ascorbic acid	Benzoyl peroxide	Chlorine dioxide
0	0	0	0	0	0
50	0	50	0	0	0
75	0	75	0	0	0
AB	AB	50	30	50	0
DXM	DXM	50	30	50	15

Animals. Weanling rats (SPF, MRC category 4) of the Wistar-derived Porton strain were supplied by Scientific Agribusiness Consultants (International) Ltd., Braintree, Essex.

Each main diet group comprised 60 males and 60 females and an additional 30 males and 30 females per group were maintained for biochemical and haematological investigations. Animals free from obvious disease and comparable in weight were acclimatized for 3 days and randomly assigned to their diet groups. They were caged in groups of three in polypropylene cages (50 cm × 32 cm × 19 cm) with stainless-steel mesh tops and floors. Cages were arranged in the racks so that each treatment group was represented as evenly as possible at each level. Room temperature was held at 22 ± 2°C and humidity at 40–50%; positive air pressure and 12 air changes/hr were maintained.

Procedure. Diets (water content approximately 12%) and water were made available *ad lib*. Diet spillage was recovered and food intakes were corrected accordingly. All animals were inspected daily for abnormal appearance or behaviour and signs of ill-health. Sick animals were isolated, to be returned to their cages on recovery or killed if their condition deteriorated. A gross autopsy was carried out on all animals unless this was precluded by cannibalism or advanced autolysis. Body weights and food intakes of individual animals were recorded weekly for the first 13 wk and monthly thereafter. Water intakes were determined monthly.

Animals surviving at 104 wk were killed by ether overdosage, any macroscopic abnormalities were noted, and brain, heart, liver, kidneys, spleen, adrenals, gonads, uterus, thyroid and pituitary were removed and weighed. Samples of these organs, and of trachea, lungs, prostate, seminal vesicles, pancreas, salivary gland, eyes, skin, skeletal muscle, cervical lymph node, thoracic aorta, oesophagus, stomach, duodenum, jejunum, ileum, colon, urinary bladder, tongue, optic nerve, sciatic nerve and spinal cord were removed and preserved in 10% buffered formalin. Stained smears of bone marrow were prepared.

Blood and urine tests. Blood for haematological and biochemical examination was obtained by cardiac puncture under light ether anaesthesia at 1, 3, 12 and 24 months. Haematological determinations (at 3, 12 and 24 months; 10 males, 10 females/diet group) comprised haemoglobin (Drabkin & Austin, 1932), packed cell volume (Strumia, Sample & Hart, 1954), erythrocyte and leucocyte counts (Coulter Electronic counter) and differential leucocyte counts.

Blood urea nitrogen (Fawcett & Scott, 1960), glucose (Keston, 1956), aspartate aminotransferase and alanine aminotransferase (Bergmeyer & Bernt, 1970), leucine aminopeptidase (Nagel, Willig & Schmidt, 1964), alkaline phosphatase (terminally only; Bessey *et al.* 1946) and total protein (Weichselbaum, 1946) were determined in the blood of five rats of each sex/group at 1, 12 and 24 months.

Urine analyses were carried out at 6, 12 and 24 months, using ten animals of each sex/group. Renal concentration and dilution tests were carried out at 6, 12, 18 and 24 months with ten rats of each sex/group.

Determination of covalently-bound bromine in adipose tissue. Fat extracted from the dried adipose tissue with light petroleum was dried and the bromine was liberated by combustion, essentially according to the oxygen flask method of Dobbs (1966). The bromine produced was absorbed in water, and the solution was made alkaline with dilute sodium hydroxide and evaporated to dryness. The residue was taken up in acid, and converted to bromoethanol by the method of Heuser & Scudamore (1970). The product was quantitatively determined by gas–liquid chromatography (Pye 104, electron capture; analytical limit 0·5–1 ppm).

Statistical analysis. Analysis of variance and covariance (Honeywell Mark III computer) and paired *t* tests were carried out. Rejection of data was controlled by statistical tests of the validity of this procedure. Dose-response relationships in diet groups 0, 50 and 75 were determined by regression analysis.

RESULTS

Appearance and behaviour

No differences in appearance or behaviour between test and control rats were noted. Abnormalities found (buphthalmos (Young, Festing & Barnett, 1974) and renal calculi) were unrelated to ingestion of the additives under test.

Mortality, weights and food intakes

Cumulative mortality data are given in Table 2. Death of one animal represents a mortality of 1·67%.

Mean body weights at various stages of the trial are listed in Table 3. "Mean body weights per day of trial" were computed and were in the range 556–579 g (males) and 330–341 g (females). In a separate trial in which growth rates and food consumption were compared for similar Wistar rats fed on the control bread diet or on diet 41B, body weights of rats

Table 2. *Cumulative mortality rate of rats given diets specified in Table 1*

Wk no.	Diet group... 0	50	75	AB	DXM
	Cumulative mortality (%)				
		Males			
29	1·7	0	1·7	0	1·7
32	1·7	0	1·7	1·7	1·7
36	3·3	1·7	1·7	1·7	1·7
52	3·3	1·7	3·3	5·0	5·0
56	5·0	1·7	3·3	10·0	5·0
76	10·0	6·7	3·3	15·7	8·3
84	16·7	13·4	6·7	18·3	11·7
92	21·7	23·3	13·4	20·0	18·3
96	21·7	31·7	13·4	23·3	20·0
104	26·7	38·3	20·0	33·3	33·3
		Females			
4	0	0	0	1·7	0
48	3·3	0	0	5·0	0
56	6·7	0	0	5·0	1·7
60	11·7	1·7	0	8·3	3·3
68	11·7	3·3	1·7	8·3	6·7
76	18·3	16·7	5·0	13·3	13·4
88	26·7	26·7	11·7	23·3	21·7
92	31·7	31·7	20·0	28·3	31·7
104	51·7	51·7	30·0	45·0	43·3

receiving the bread diet were higher after 100 days on trial than those of the rats feeding on a standard rat diet (41B meal) by about 2·5% (males) and 11% (females).

Table 4 gives the food consumption data. Normalized food intakes ranged from 35·8 to 36·6 g/kg body weight/day for males and from 47·3 to 48·5 g/kg/day for females. The (bromate-derived) bromide intake levels (expressed as mg bromine/kg/day) were thus: groups 50, AB and DXM, males, 0·517, females 0·686; group 75, males 0·765, females 1·015. Food intakes of Wistar rats fed the control bread diet were about 11% (males) and 12·7% (females) lower than those of similar rats consuming diet 41B, in the separate trials mentioned earlier. Water intakes (ml/kg/day) ranged from 48 to 61 (males) and from 103 to 115 (females). Male controls drank more water than males in the test groups but the reverse effect was seen in females.

Haematology

In the male rats, there were no significant differences, in the parameters measured, between animals in test groups and those in group 0 after 3 and 12 months. At 24 months, red cell counts and PCVs of group 50 rats were lower than those of the controls ($P < 0·05$), and PCV was also lower than the control in group 75. All other differences were not significant. There were no significant differences in haematological parameters at any stage of the trial between females fed on test and control diets.

Table 3. *Body weights of rats given diets specified in Table 1*

Diet group	Mean body weight (g) at wk no. 0	2	12	25	36	48	60	72	84	104
					Males					
0	76	167	412	489	547	587	628	648	663	652
50	78	173	431**	514**	577**	619	655*	675*	688	645
75	80	172	422	499	557	599	635	661	674	641
AB	77	169	422	497	556	598	633	650	675	646
DXM	77	170	422	502	562	603	639	662	681	654
					Females					
0	74	138	253	290	314	335	384	408	427	407
50	73	136	250	285	307	329	371	395	402	377*
75	73	136	251	285	313	332	371	408	431	416
AB	74	137	252	287	311	334	377	406	424	390
DXM	72	136	248	281	303	323	362	390	418	404

Values marked with asterisks differ significantly from those of the controls (*$P < 0·05$; **$P < 0·01$).

Table 4. *Mean food intake data of rats given diets specified in Table 1*

Diet group	Mean food consumption (g/day) at wk no.			
	1	6	52	100
		Males		
0	12·2	20·0	18·4	19·3
50	12·6	21·3	19·2	19·3
75	12·6	20·3	18·4	17·9
AB	12·3	20·7	18·5	19·4
DXM	12·7	20·4	18·6	18·1
		Females		
0	11·0	15·2	15·9	15·4
50	10·9	15·2	15·7	16·2
75	11·1	15·2	15·1	15·5
AB	11·1	15·1	15·5	15·5
DXM	11·2	14·8	15·7	16·9

Blood analyses

Dose-related reductions in blood glucose levels were noted in both males and females at 24 months, but at no other stage of the trial. There were no significant differences in other biochemical parameters during the trial.

Renal function tests, urine analyses

In conditions of normal water intake, there was a statistically significant trend ($P < 0{\cdot}025$) to lower urine specific gravity in the males at 12 months as the bromate treatment level increased from 0 to 75 ppm and the value at 75 ppm was significantly lower than the control ($P < 0{\cdot}05$). The simultaneous trend to increased urine volume only reached the 10% level of significance. The corresponding values at 24 months did not differ significantly. No significant treatment-related differences in the specific gravity of the urine were observed for the females at either stage of the trial.

The specific gravity of the urine passed 16–20 hr after water-loading by females of group AB at 24 months was significantly lower than the control value, but this effect was not apparent at any other stage of the trial for either sex. Otherwise none of the various urine parameters monitored, including urinary aspartate aminotransferase, protein, glucose and cellular elements, provided any evidence of treatment-induced effects.

Post-mortem findings

The number and types of abnormality seen did not differ significantly between the diet groups. Organ weight data are shown in Tables 5 and 6.

Histopathology

The histopathological findings are set out in Table 7. The principal site of tumours was the pituitary; malignant tumours occurred in pancreas (1), skin (3), connective tissue (3), reticuloendothelial system (17 males, 18 females; no significant differences between diet groups) and mammary gland (5). Differences in the incidence of benign or of malignant tumours between diet groups were not statistically significant.

Total dietary and covalently-bound bromine in rat adipose tissue

Samples of flours 0, AB and DXM each contained 4 ppm bromine; breadcrumb of diets 0, 50, 75, AB and DXM contained 5, 25, 38, 29 and 25 ppm bromine respectively; the corresponding diets gave values of 5, 27, 29, 20 and 22 ppm bromine respectively, the other ingredients causing a lowering of sensitivity and analytical precision for these samples.

Table 5. *Absolute organ weights after 104 wk on diets specified in Table 1*

Diet group	Group mean organ weights (g)										Terminal body weights†
	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Thyroid	Pituitary	Uterus	
					Males						
0	2·26	1·90	18·67	4·30	1·76	0·07	3·33	0·033	0·031	—	601
50	2·34	2·01	18·07	4·18	1·67	0·16	3·06*‡	0·038	0·027	—	587
75	2·30	1·95	18·43	4·41	1·53	0·09	3·24	0·034	0·023	—	606
AB	2·29	1·98	17·66	4·32	1·55	0·10	3·40	0·034	0·033	—	596
DXM	2·32	1·92	17·62	4·24	1·77	0·08	3·33	0·035	0·025	—	597
					Females						
0	2·02	1·55	13·54	2·96	1·11	0·08	0·10	0·027	0·067	0·80	349
50	2·08*‡	1·46	13·00	3·10	1·10	0·09	0·10	0·027	0·110*‡	0·88	329
75	2·05	1·47	13·23	2·94	1·12	0·07	0·10§	0·027	0·097	0·83	364
AB	2·04	1·54	12·66	3·01	1·19	0·07	0·13	0·027	0·102	0·85	346
DXM	2·06	1·42	14·13	2·84	1·24	0·09	0·12	0·027	0·087	1·12*∥	359

Values marked with asterisks differ significantly from those of controls ($*P < 0{\cdot}05$).
†In females, the weights of animals in group 50 differed significantly from those in groups 75 and DXM ($*P < 0{\cdot}05$) but not from those in control or AB groups.
‡Not significant by Tukey test.
§One outlier rejected: outliers have not been rejected in other Tables unless so stated.
∥Rejection of extreme outliers using a criterion of 99·5% confidence makes this mean 0·86 (SD 0·36), not significantly different from those of the other groups, including the control group.

Table 6. *Relative organ weights after 104 wk on diets specified in Table 1*

Diet group	Group mean organ:body weight ratios (%)									
	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Thyroid	Pituitary	Uterus
				Males						
0	0·39	0·33	3·16	0·73	0·30	0·013	0·56	0·006	0·006	—
50	0·42	0·35	3·05	0·74	0·28	0·025	0·53	0·007	0·005	—
75	0·39	0·33	3·08	0·76	0·26	0·015	0·54	0·006	0·005	—
AB	0·40	0·35	2·99	0·75	0·27	0·016	0·56	0·006	0·006	—
DXM	0·41	0·33	2·98	0·74	0·29	0·014	0·57	0·006	0·005	—
				Females						
0	0·61	0·46	3·92	0·88	0·33	0·02	0·03	0·008	0·022	0·24
50	0·66*	0·46	4·05	0·99†	0·35	0·03	0·03	0·009	0·035	0·27
75	0·60	0·42	3·77	0·85	0·32	0·02	0·06	0·008	0·030	0·24
AB	0·62	0·46	3·74	0·90	0·35	0·02	0·04	0·008	0·033	0·25
DXM	0·60	0·41	3·97	0·83	0·34	0·03	0·03	0·008	0·028	0·31

*This value differs significantly from the control, AB and DXM values ($P < 0·05$), but not from the group-75 value.
†This value differs significantly from the group-75 and DXM values ($P < 0·05$).

Analysis of adipose tissue from rats in groups 0, 75, AB and DXM showed bromine levels to be below the limit of quantitative determination (0·5–1 ppm) in all groups. There was thus no retention or accumulation of significant amounts of covalently-bound bromine in the adipose tissue of the rats receiving the test diets.

DISCUSSION

Potassium bromate oxidizes sulphydryl groups in the flour proteins to disulphides and is concomitantly reduced to the bromide which remains in the bread. The half-life of bromide administered to rats varies inversely with the level of chloride in the diet (Rauws & Van Logten, 1975), and since the ratio of chloride (in salt) to bromide in bread made from flours treated with 50 ppm potassium bromate is of the order of 250:1 in normal commercial recipes, rapid elimination of bromide is assured.

Behavioural abnormalities (Van Logten, Wolthuis, Rauws & Kroes, 1973) and growth retardation, increased weights (relative to body weight) of thyroid and adrenals and decreased relative prostate weights were previously observed in rats fed for 90 days on diets containing up to 19,500 ppm sodium bromide (Van Logten, Wolthuis, Rauws, Kroes, Den Tonkelaar, Berkvens & Van Esch, 1974). None of these effects were evident in the present 2-yr trial which involved much lower concentrations of bromide; massive doses of a potent sedative might be expected to produce some of the behavioural effects observed in the earlier work.

Survival rates in the male animals at 104 wk were excellent, and the slight differences observed were in general unrelated to the additives being consumed; survival was significantly higher ($P < 0·05$) on the 75 ppm bromate-containing diet than on the other diets, however. Dose-related improvement in survival ($P < 0·05$) was noted in the females, among whom deaths were more frequent than in the males, in part because of the greater susceptibility of the females to develop pituitary tumours (Table 7; Ribelin & McCoy, 1965; Wolfe, Bryan & Wright, 1938).

Use of the additives caused no loss in palatability or nutritive value of the diets, as shown by the very small differences between diet groups in food intakes (1–2%) and body weights (3–4%). However, there were slight differences between control and test groups in water consumption, although these differences were reversed in the opposite sexes.

The neutrophilia reported by Van Logten *et al.* (1974) and reflected in our own trial with mice (Ginocchio *et al.* 1979) was not shown in the present trial, and no consistent differences were observed in other haematological parameters.

At month 24 only, blood-sugar values in both males and females were significantly lower in groups 50 and 75 than in the corresponding controls, while the values for the two remaining groups, also containing 50 ppm bromate, were not significantly different from the controls. However, the opposite result was seen in the study with mice (Ginocchio *et al.* 1979), and in view of these inconsistencies, the effects can probably be disregarded.

Post-mortem findings, including organ-weight data expressed as such or relative to body weight, convey no suggestion of hazard associated with the use of the additives under test. These findings were confirmed by the histopathological examination of the rat tissues, which showed no evidence either of damage due to the ingestion of toxic materials or of carcinogenicity attributable to the additives being tested.

No accumulation of covalently-bound bromine was found to occur in the adipose tissue of the rats in this 2-yr study.

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Table 7. *Incidence of histopathological findings after 104 wk on diets specified in Table 1*

	Males					Females				
Affected organ and/or type of lesion — Diet group...	0	50	75	AB	DXM	0	50	75	AB	DXM
No. examined...	88	90	88	85	90	89	89	88	89	88
Kidneys										
Glomerulonephrosis	81	74	74	78	79	80	78	78	81	77
Calculi or calcium deposits	3	4	3	6	9	63	35	39	45	39
Other lesions	8	2	3	2	2	4	4	1	1	1
Liver										
Cell vacuolation	46	58	44	47	53	63	43	53	38	57
Leucocytic foci & leucocytic infiltration	19	8	14	13	7	28	23	19	17	26
Foci or areas of necrosis	5	6	2	1	4	10	7	5	2	1
Other lesions	11	20	18	21	23	44	62	55	36	26
Heart										
Interstitial fibrosis	32	46	33	48	63	41	22	20	14	33
Other lesions	8	10	2	6	2	7	6	4	3	4
Lungs										
Pneumonitis or pneumonia	40	50	19	25	27	22	42	16	26	17
Other lesions	35	50	37	32	33	29	31	16	20	17
Spleen										
Increased haematopoiesis	11	19	10	19	23	14	10	24	28	35
Thyroid										
Various minor lesions	7	7	3	5	5	2	3	1	2	0
Pancreas										
Pancreatitis	4	0	2	2	0	2	2	3	0	0
Periarteritis	2	9	12	5	12	9	15	10	14	16
Giant islet	0	2	0	0	1	2	1	0	0	0
Hyperplastic nodule	0	0	0	1	0					
Pituitary										
Cyst	1	3	1	2	3	1	0	0	1	0
Hyperplasia	7	3	0	2	1	3	4	2	7	0
Testes										
Periarteritis	8	8	8	5	1					
Atrophy	29	14	19	14	6					
Prostate, seminal vesicles										
Inflammatory reaction	17	6	8	5	9					
Bladder										
Inflammatory changes	8	5	3	3	5	0	0	0	1	0
Ovaries										
Cystic						15	8	8	5	3
Atrophy or partial atrophy						17	11	5	5	7
Uterus										
Cystic endometrial hypertrophy or cystic dilatation						17	8	6	8	14
Pyometra or gross inflammatory reaction						0	2	0	2	1
Adrenals										
Various ageing pathology	23	35	35	41	46	47	42	71	55	66
Stomach										
Various minor lesions	39	33	23	26	34	23	25	16	21	28
Tumours										
Benign	33	42	33	31	34	43	60	50	43	41
Malignant	4	6	4	3	6	6	3	3	4	8
Total	37	48	37	34	40	49	63	53	47	49

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LONG-TERM TOXICITY AND CARCINOGENICITY STUDIES OF THE BREAD IMPROVER POTASSIUM BROMATE
2. STUDIES IN MICE*

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Abstract—Mice were fed for 80 wk on five bread-based diets in which the bread was prepared from untreated flour or from flour treated with 50 or 75 ppm potassium bromate or with one of two mixtures of potassium bromate with other commonly-used additives. Appearance, behaviour, health and survival were similar in test and control groups. No carcinogenic effects were produced by any of the diets. Anaemia was present in all male groups (including controls), except the group receiving 50 ppm bromate together with three other flour additives, and in females terminally. There was a dose-related decrease in the red blood cell count of males at 3 months and neutrophilia seen at 12 and 18 months was dose-related. Raised blood-sugar levels, related to dose were found in females at 3 and 12 months, but the effect was not significant at 18 months, and was not found at any stage in the males. Dose-related differences in the weights of the heart, the pituitary and the uterus were found; when expressed relative to body weight, values for the heart and the uterus were no longer dose-related, but the pituitary, the brain, the kidneys and the thyroid showed significant dose-related trends. These effects were not associated with pathological changes in the structures of the tissues concerned.

INTRODUCTION

Studies in rats of the oxidative bread improver potassium bromate and two mixtures of this compound with other common flour and bread improvers are described in the preceding paper (Fisher, Hutchinson, Berry, Hardy, Ginocchio & Waite, 1979). The present paper describes the results of a study of similar design, using mice.

EXPERIMENTAL

Materials and methods were as described in the preceding paper. Mice of the Theiller's Original strain (900) were obtained from A. Tuck & Son, Rayleigh, Essex and housed in groups of five in an animal room kept at 21 ± 2°C and 40–50% relative humidity at Consultox Laboratories, under the supervision of their staff. Experimental protocols, including diet formulation and supply, and overall project control were the responsibility of FMBRA. As in the rat study, the five bread-based diets were made up using flour containing: no bromate (0), 50 ppm bromate (50), 75 ppm bromate (75), 50 ppm bromate, 30 ppm ascorbic acid and 50 ppm benzoyl peroxide (AB), or 50 ppm bromate, 30 ppm ascorbic acid, 50 ppm benzoyl peroxide and 15 ppm chlorine dioxide (DXM). Details of experimental design are as described for the rat study, except that the duration of the trial was 80 wk and the mice were weighed weekly for the first 2 months and monthly thereafter, the mean individual weight for each group being recorded.

RESULTS

General condition appeared to be good in control and test groups at all stages and no abnormal behaviour was noted. Moribund individuals were rarely observed. Cumulative mortality data are given in Table 1. The differences in mortalities were not significant statistically. Group mean body weights are given briefly in Table 2 and show no significant differences at any stage of the trial.

Food intakes are presented in Table 3. Differences between the groups were small and within the range of variation usually found in mice of this strain, sex and age. Food intakes were lower than those usually observed with this strain of mice when fed on stock commercial diets, but were not affected by the use of the additives, being due to the higher nutritional value of the bread-based diets. 'Normalized' (weighted

*Full details and an extended discussion of the results are available on loan from FMBRA.

Table 1. *Cumulative mortality rate of groups of 60 mice given diets based on bread made from flours treated with bromate and other additives*

Diet group	Cumulative mortality (%) at month ...					
	4	10	12	14	15	18·5
Males						
0	0	0	1·7	18·3	26·7	65·1
50	0	1·7	6·7	13·4	26·7	65·1
75	0	1·7	1·7	3·3	16·7	61·8
AB	0	0	1·7	15·0	25·1	53·4
DXM	0	1·7	1·7	16·7	25·1	58·5
Females						
0	1·7	1·7	3·3	8·4	15·0	56·8
50	1·7	1·7	1·7	1·7	11·7	48·4
75	0	0	0	1·7	26·7	56·8
AB	0	0	0	3·3	15·0	63·5
DXM	0	0	1·7	10·0	20·0	53·4

Table 3. *Mean food intake data of groups of 60 mice given diets based on bread made from flours treated with bromate and other additives*

Diet group	Mean food consumption (g/wk) at wk no....			
	1	13	52	78
Males				
0	39	42	33	43
50	44	43	33	45
75	44	43	35	47
AB	38	39	32	39
DXM	39	40	33	39
Females				
0	52	41	30	39
50	50	41	29	42
75	57	44	28	43
AB	49	38	29	42
DXM	41	48	31	40

mean) bromine intakes derived from potassium bromate were 0, 1·76, 2·64, 1·70 and 1·63 mg/kg body weight/day in males and 0, 2·03, 2·99, 1·99 and 2·08 mg/kg/day in females for groups 0, 50, 75, AB and DXM respectively.

Results of the haematological studies for males are given in Table 4. Anaemia was prevalent in the males of all groups (including controls) and in the females at 18 months. No dose-related differences were observed in the females, and individual statistically significant differences did not follow any pattern with respect to the stage of the trial or the dose.

No dose-related differences in blood biochemistry were found in male mice throughout the trial. In the females, dose-related increases ($P < 0·01$) in blood-glucose levels were observed at 1 and 12 months but not at 18 months, when none of the values differed significantly from that of the control.

Renal concentration and dilution tests and urine analyses showed that the kidneys of animals in all groups were functioning normally at the time of each test. Urinary glutamic–oxaloacetic transaminase was higher in test groups than in the controls in males but not consistently so in females; in particular the

Table 2. *Body weights of groups of 60 mice given diets based on bread made from flours treated with bromate and other additives*

Diet group	Mean body weight (g) at wk no....			
	0	13	52	80
Males				
0	20	43	50	44
50	20	44	51	42
75	20	42	49	41
AB	20	42	46	42
DXM	20	40	50	42
Females				
0	19	34	41	35
50	19	36	44	36
75	19	33	42	37
AB	19	36	41	37
DXM	19	35	41	38

value for group 75 equalled that of the control. Urinary cell counts showed no differences between the diet groups.

The few gross abnormalities observed were of the type expected for aged mice of this strain, and their numbers were similar in test groups and controls. Organ weights and organ weights relative to body weights are given in Tables 5 & 6. Histopathological findings are summarized in Table 7.

Samples of adipose tissue from mice in groups 0, 50 and 75 (2–5 mice of each sex/group) were analysed for bromine as described in the preceding paper (Fisher *et al.* 1979). Small amounts of bromine were detected only in samples from males of groups 50 and 75 (1 ppm) and females of group 50 (2 ppm).

DISCUSSION

The growth, food intake and health of the mice remained satisfactory throughout the trial, and the animals showed no evidence of the failure of grooming, and motor incoordination observed in rats by Van Logten, Wolthuis, Rauws & Kroes (1973) using very high doses of sodium bromide. There were indications of the neutrophilia seen by Van Logten, Wolthuis, Rauws, Kroes, Den Tonkelaar, Berkvens & Van Esch, (1974) in the dose-related trends shown by the male mice at 12 and 18 months, but the opposite effect was seen at 3 months and no effect was found at any stage in the females. The dose-related lowering of red cell counts at 3 months in the males was absent at 12 and 18 months, and did not occur in the females at any stage. Anaemia was present in the majority of males in all groups (including the controls) except DXM, and in the females at 18 months; no cause was established. Dose-related increases in the (non-fasting) blood glucose values of the females were observed at 3 and 12 months, but did not attain the 5% level of significance at 18 months, and were not seen in the males. A trend to lower total plasma protein with dosage was seen at 12 months in the females only, and at no other stage, and was not seen in the males; group 50 males were indeed significantly higher in total plasma protein than the controls at 18 months, while the group 75 males had similar

Table 4. *Mean haematological data for groups of 10 male mice given diets based on bread made from flours treated with bromate and other additives*

Month	Dietary group	Hb (g/100 ml)	RBC ($10^6/mm^3$)	PCV (%)	Leucocytes: Total ($10^3/mm^3$)	Differential (%): N	L	M	E
3	0	12·2	**8·4	42	10·2	*16	82	1·6	
	50	12·4	7·2*	42	9·3	17	81	1·7	
	75	12·4	7·1**	42	9·8	10*	89*	0·9	
	AB	12·1	7·0**	40	5·7*	11*	88*	0·6	
	DXM	12·5	8·0	43	10·5	11*	87	1·7	
12	0	7·8	4·8	25	17·5	***28	**72	*0·4	
	50	9·1	5·0	28	17·8	37*	60*	3·4**	
	75	9·9	4·3	31	13·0	44**	54**	1·8	
	AB	11·4*	5·5	34*	16·2	40*	58*	2·2	
	DXM	12·3**	6·0	37**	15·2	39*	58*	2·0	
18	0	11·2	6·5	33	13·0	*17	81	1·8	0·8
	50	11·2	6·3	33	17·9	20	78	1·7	1·3
	75	11·0	6·3	33	18·6	31	67*	1·0	0·4
	AB	11·6	6·9	35	13·4	23	72	3·3	1·5
	DXM	9·5	5·4	28	22·1	29	69	1·4	0·9

Hb = Haemoglobin RBC = Red blood cells PCV = Packed cell volume
N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils
Vertical lines joining values indicate a statistically significant dose-relationship: the asterisks above the line indicate the level of significance of this relationship.
Single values marked with asterisks differ significantly from the control value (*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$).

Table 5. *Organ weights of survivors from groups of 60 mice fed diets based on bread made from flours treated with bromate and other additives for 80 wk*

Dietary group	Organ weights†										Terminal body weights
	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Thyroid	Pituitary	Uterus	
Males											
0	0·45	*0·35	3·29	0·77	0·54	0·020	0·24	7·65	**3·13	—	42·7
50	0·47	0·32	3·33	0·80	0·45	0·019	0·20	9·26	2·32	—	40·8
75	0·45	0·29	2·90	0·85	0·43	0·020	0·20	11·00*‡	1·55	—	41·7
AB	0·46	0·30	2·72	0·76	0·35	0·016	0·20	10·35*‡	2·71	—	38·9
DXM	0·54	0·31	3·07	0·79	0·44	0·020	0·21	12·50*	1·52	—	42·1
Females											
0	0·45	0·25	2·49	0·61	0·55	0·017	0·07	9·65	1·64	*0·46	34·2
50	0·46	0·25	2·57	0·65	0·43	0·022	0·10	10·48	1·67	0·67	35·4
75	0·45	0·25	3·00	0·62	0·50	0·020	0·08	9·53	2·27	0·66	36·3
AB	0·46	0·27	2·33	0·64	0·38	0·020	0·09	8·71	1·82	0·60	34·7
DXM	0·47	0·27	2·66	0·68	0·46	0·018	0·09	10·07	1·58	0·62	35·6

†Organ weights are expressed in grams with the exception of those of the pituitary and thyroid, expressed in milligrams.
‡Not significant by Tukey's test.
Vertical lines joining values indicate a statistically significant dose-relationship; the asterisks above the line indicate the level of significance of this relationship.
Single values marked with asterisks differ significantly from the control value (* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$).

Table 6. *Relative organ weights of survivors of groups of 60 mice fed diets based on bread made from flours treated with bromate and other additives for 80 wk*

Dietary group	Mean organ: body weight ratios (%)									
	Brain	Heart	Liver	Kidneys	Spleen	Adrenals	Gonads	Thyroid	Pituitary	Uterus
					Males					
0	*1·00	0·79	7·22	**1·72	1·12	0·045	0·55	**0·017	***0·007	—
50	1·09	0·75	7·77	1·87	1·06	0·044	0·46	0·022	0·005*	—
75	1·11	0·70	6·97	2·05*	1·07	0·049	0·47	0·027*	0·004**	—
AB	1·17	0·74	6·77	1·89	0·91	0·041	0·50	0·026	0·007	—
DXM	1·30	0·75	7·28	1·87	1·05	0·047	0·51	0·031**	0·004**	—
					Females					
0	1·33	0·74	7·30	1·80	1·59	0·050	0·21	0·028	0·005	1·38
50	1·30	0·68	7·10	1·80	1·17	0·061	0·27	0·029	0·005	1·79
75	1·24	0·70	8·36	1·71	1·40	0·055	0·21	0·026	0·006	1·76
AB	1·35	0·78	6·68	1·85	1·09	0·060	0·26	0·026	0·006	1·71
DXM	1·30	0·74	7·23	1·86	1·27	0·072	0·24	0·027	0·004	1·70

Vertical lines joining indicate a statistically significant dose-relationship; the asterisks above the line indicate the level of significance of this relationship.
Single values marked with asterisks differ significantly from the control value (* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$).

Table 7. *Principal histopathological findings in mice fed diets based on bread made from flours treated with bromate and other additives for 80 wk*

Affected organ and type of lesion	Males					Females				
Diet group...	0	50	75	AB	DXM	0	50	75	AB	DXM
No. examined...	35	46	53	47	49	53	54	52	49	51
No. autolysed*...	(9)	(18)	(22)	(13)	(15)	(19)	(13)	(15)	(19)	(12)
Generalized amyloid (Principal organs affected: adrenals, intestines, kidney, liver, heart, spleen, stomach, ovaries and uterus)	32	38	49	46	48	47	50	47	45	47
Liver										
Minor lesions other than amyloid	21	25	23	18	20	43	37	36	27	31
Kidney										
Glomerulonephrosis (including cystic glomeruli and fibrosis)	10	6	14	11	16	25	20	18	16	22
Other lesions	1	5	3	2	0	6	2	9	1	5
Lungs										
Pneumonitis	3	4	7	2	0	12	7	7	2	7
Other lesions	2	8	3	4	4	9	14	8	4	7
Heart										
Focal myocarditis, calcification or pericarditis	2	2	2	0	0	3	2	4	2	3
Spleen										
Increased haematopoiesis	0	4	2	4	3	10	6	3	6	9
Lymphoid hyperplasia	1	2	1	0	0	0	2	0	0	0
Testes										
Atrophy, partial or complete	9	14	11	18	13					
Uterus										
Cystic endometrial hyperplasia						24	32	31	25	26
Ovaries										
Cystic						5	8	20	11	8
Neoplasms										
Generalized lymphosarcoma	1	1	0	1	0	1	0	3	0	0
Myeloid leukaemia	0	0	0	0	1	1	0	0	0	0
Lung adenoma	1	3	3	3	1	2	8	2	2	4
Peritoneal lipoma	0	1	0	0	0					
Thymic lymphosarcoma						0	0	0	0	1
Reticulum cell neoplasm						2	0	1	0	3
Ovary adenoma						0	0	0	2	0
Salivary gland adenoma						0	0	1	0	0
Subcutaneous lipoma						0	0	0	0	1
Splenic haemangioma						0	0	1	0	0
Hepatic cavernous haemangioma						0	1	0	0	0
Uterine fibroma						0	0	0	0	1
Benign tumours	1	4	3	3	1	2	9	4	4	6
Malignant tumours	1	1	0	1	1	4	0	4	0	4
Total tumours	2	5	3	4	2	6	9	8	4	10

*These animals were given a minimal examination.
Differences between the groups are not statistically significant by the Chi-squared test.

values to the controls. It is doubtful whether such inconsistent trends are of toxicological significance.

In view of the evidence of normal renal function at all stages of the trial, the absence of raised urinary glucose levels and the lack of differences between controls and treatment groups in kidney histopathology, it seems unlikely that the raised UGOT values in the test groups at 18 months indicate nephrotoxicity of the additives.

Significant dose-related differences between control and test groups in heart, pituitary and uterus weights were found at post mortem. When expressed relative to body weight, the relationship was absent in the cases of the uterus and heart, but still highly significant for the pituitary, and new dose-response relationships in brain, kidneys and thyroids, the weights of which increased relative to body weight with increasing dose. However, though particular attention was directed to the endocrine glands in view of the work of Van Logten *et al.* (1974), no histopathological differences were found between test and control groups, and this was also true for the other organs in which weight differences were observed.

The main histopathological findings, of amyloid deposition in various tissues, occurred equally in controls and test groups, and may possibly have been influenced by the nature of the basal diet containing 79% bread, though there is no evidence on this point and the animals appear to have been particularly susceptible to this condition. Other lesions and abnormalities were those commonly seen in aged mice and were evenly distributed in test groups and controls.

There is no evidence that any of the treatments affected the incidence of neoplasms, which were of the type usually seen in mice. Malignant tumours were only found in the reticuloendothelial system, and were those commonly seen in mice—lymphosarcoma, reticulum cell neoplasm and myeloid leukaemia. As often occurs, they were rather more frequent in females than in males, but their overall incidence was as high in control mice as in those receiving the test diets.

There was thus no evidence that any of the flour treatments in any way affected the incidence of neoplastic or non-neoplastic histopathological changes in this study.

No significant dose-related accumulation of covalently-bound bromine occurred in the mouse after 18 months' feeding on diets containing 79% bread made from flour treated with up to 75 ppm potassium bromate. The results accord well with those obtained in rats (Fisher *et al.* 1979) and indicate that no hazard of the type found with brominated vegetable oils is associated with the use of potassium bromate at concentrations in the range 0–75 ppm.

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PHYSIOPATHOLOGY OF HAEMORRHAGIC SYNDROME RELATED TO OCHRATOXIN A INTOXICATION IN RATS

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Abstract—Wistar rats were given by gavage a daily 4-mg/kg dose of ochratoxin A over 4, 6, 8 or 10 days. Studies on haemostasis showed a drop in plasma fibrinogen and decreases in factors II, VII and X, and in the thrombocyte and megakaryocyte counts. The possibility of a disseminated intravascular coagulation syndrome is discussed and it is suggested that an effective fall in the vitamin-K pool caused ochratoxin A to have an indirect effect similar to that of coumarin. Blood analyses showed a later haemoconcentration related to an increase in neutrophils, which was probably due to the presence of necrotic foci in the organs of the treated animals.

INTRODUCTION

Acute toxicity tests (Galtier, Moré & Bodin, 1974) and short-term studies (Galtier, Bodin & Moré, 1975) in rats demonstrated the appearance of numerous haemorrhagic foci during the last stages of ochratoxin A intoxication. Even though these signs have been found in all species studied, their further investigation has been undertaken only by Doerr, Huff, Tung, Wyatt & Hamilton (1974) and Doerr, Huff & Hamilton (1976). These authors found that the mycotoxin caused significant increases in recalcification and prothrombin times in young broiler chickens (Doerr *et al.* 1974) and reduced the concentration of haemostasis factors (Doerr *et al.* 1976).

Because of the similarity between ochratoxin and anticoagulants of the coumarin series, it seemed advisable to study the physiopathology of the haemorrhagic syndrome associated with ochratoxin A intoxication in rats. The present investigation was undertaken to explore haemostasis and haematological factors in rats receiving oral doses of the toxin for different periods of time.

EXPERIMENTAL

Animals and diets. Male Wistar rats, weighing 235·8 ± 7·5 g, were divided randomly into a control group of 28 rats and four test groups each of 14 animals. The controls were caged (at an ambient temperature of 20–22°C) in groups of four and the test rats in pairs, and all the animals were given UAR feed and water *ad lib.*

Dosage. Ochratoxin A in doses of 4 mg/kg/day was administered by oesophageal intubation in an isotonic solution of sodium bicarbonate (14 g/litre) to the four test groups for 4, 6, 8 or 10 days. The dosage volume of bicarbonate solution was administered to the control groups.

Sampling. Blood (5-ml samples) was collected from the abdominal aorta of anaesthetized animals into tubes containing 0·5 ml 0·0106 M-sodium citrate and the plasma was separated by centrifuging (2000 g) at 25°C.

Analyses and cell counts. The fibrinogen in 0·5 ml plasma was precipitated by adding 10 ml thrombin (2 Mellenby units/ml of 0·15 M-sodium chloride) and the fibrin clot was isolated after a 10-min incubation (37°C). It was then washed three times with a solution of 0·15 M-sodium chloride and lastly was dissolved in 1 ml 1 N-sodium hydroxide. The fibrinogen levels were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The specific activities of clotting factors II (prothrombin), V (proaccelerin) and VII + X (proconvertin-Stuart factor) were measured by standard methods (Caen, Larrieu & Samana, 1975). Thrombocyte counts and mean thrombocyte volumes were estimated by the Coulter-counter method as previously described (Boneu, Corberand, Plante & Biermé, 1977).

Megakaryocyte counts were made on longitudinal sections of undecalcified femur to preserve the integrity of marrow cells. The bone epiphyses were removed with a fine saw and samples were dehydrated by processing with *tert*-butanol in an oven at 37°C for 2 days. Tissues were impregnated with polyester resin (Stratyl A 116 from Polymir, Bordeaux), by transfer to increasing concentrations of the resin in styrene (10%, v/v, resin for 48 hr, 25% resin for 24 hr and then 100% resin for 48 hr). During the final period, a vacuum of approximatively 10 mm Hg was applied overnight to remove any bubbles. First embedding was then carried out in fresh 100% resin in a silicon mould. One drop of catalyst (Polyca X 8) was added and the block was allowed to polymerize in an oven at 60°C for 3 days. When moderately hard, the block was moved to a new mould and re-embedded in fresh resin with added catalyst and hardener (Polyax Y 3). After polymerization for 24 hr at 40°C, the block was trimmed to 15 × 5 × approxi-

mately 10 mm with a fine saw and then gently trimmed, using a rotary Yung microtome fitted with a tungsten carbide knife, until the full face of the bone diaphysis was exposed. The 9-μm sections obtained were stained with a solution of 1% toluidine blue in 75% ethanol. Ten slides were viewed for each animal with a Reichert Visopan. The frosted glass of the Visopan had previously been divided into squares varying in surface area according to the objective used. Megakaryocyte counting was similar to blood-smear counts and results were expressed as the arithmetic means of the different slides examined.

Plasma-bilirubin levels were measured by means of the Biochemica Test Combination (Boehringer Mannheim, ref. 15944). Total leucocyte counts, erythrocyte counts, mean erythrocyte volumes, packed cell volumes, haemoglobin concentrations and haemoglobin ratios were obtained with the use of an electronic Coulter-counter device. A differential white cell count was carried out on all blood samples using the May-Grünwald–Giemsa stain. The search for fibrin deposits was carried out in sections of kidney, lung and digestive tract stained with Mallory's phosphotungstic-haematoxylin.

Statistical analysis. These experimental data were analysed first by two-way analysis of variance without interaction: one 7-level factor 'group' and one 5-level factor 'treatment'. In order to take the ordered structure of the levels of 'treatment' factor into consideration, the statistical analysis was continued with multiple comparison of means of the 'treatment' factor and with a further study of the four following contrasts:

C1 = 4(Controls) – (4 days group [d-gp] + 6 d-gp + 8 d-gp + 10 d-gp)
C2 = 3(Controls + 4 d-gp) – 2(6 d-gp + 8 d-gp + 10 d-gp)
C3 = 2(Controls + 4 d-gp + 6 d-gp) – 3(8 d-gp + 10 d-gp)
C4 = (Controls + 4 d-gp + 6 d-gp + 8 d-gp) – 4(10 d-gp)

This approach did, in fact, make possible the classification of the factor treatment levels and also the visualization of the kinetics of the appearance of disturbance In the last two statistical studies, the null difference hypothesis was tested by Bonferroni's method (Morrison, 1976), in which the differences are considered as a whole and not individually in the 5% level test.

RESULTS

Clinical observations in rats treated with ochratoxin A have already been described (Galtier *et al.* 1975). The most significant dose-related effects were loss of body weight, a reduction in spontaneous activity, hypothermia, cachexia and diarrhoea. The mean results of the biochemical and haematological studies in the test groups are compared with those in the controls in Table 1.

According to the analysis of variance, daily administration of ochratoxin A did not affect the factor V (proaccelerin) concentration, the mean erythrocyte volume or the numbers of eosinophils, lymphocytes and monocytes. Interference with the haemostatic mechanism was marked by a fall in plasma fibrinogen (–87% in 10 days) and by a decrease in prothrombin, in proconvertin-Stuart factor (factors VII + X) and in thrombocyte and megakaryocyte counts.

Histological study of sections of kidney, lung and digestive tract revealed no fibrin deposits, but the possibility of a disseminated intravascular coagulation cannot be dismissed altogether because such deposits may be transient.

The haematological investigations demonstrated parallel increases (of between 23 and 27% after 10 daily doses of ochratoxin) in packed cell volumes, haemoglobin concentrations and erythrocyte counts. A 70% increase in total leucocytes after the 10-day ochratoxin treatment was due to a fourfold rise in neutrophils. In addition, there was a roughly threefold increase in bilirubin concentration in the 10-day period.

DISCUSSION

The multiple comparison of means seldom gives rise to well-differentiated groups of values because of the conservative nature of the method used. The method does, however, allow the parameters to be grouped on the basis of the changes observed. Thus there was a strong correlation in this study between packed cell volumes, haemoglobin concentrations and erythrocyte counts and also between the total white cell and neutrophil counts. There was another strong correlation between the rise in the thrombocyte volume and the decrease in thrombocyte count. The contrast analysis results, however, demonstrate the progressive character of the toxin's action on each of the parameters considered. When the values were plotted against time, the effects on the haematological parameters, observed after 6–8 days, were preceded by the changes in fibrinogen concentration and platelet counts, which were clearly apparent by days 4–6.

An alteration in the haemostatic mechanism is demonstrated by the fall in fibrinogen level, the decrease in thrombocyte counts and the anti vitamin K-like response of the haemostatic factors II, VII and X.

The drop in plasma fibrinogen could have been due either to a decrease in hepatic synthesis or to an increase in its assimilation as a result of a disseminated intravascular coagulation syndrome.

The first hypothesis is supported by the significant increase in the plasma levels of bilirubin unaccompanied by icterus or signs of bile-duct obstruction. This effect could have been due to changes in liver cell morphology, which were identified in a parallel histopathological examination showing late changes in the hepatocytes, notably karryorhexis, pycnosis and cytoplasmic homogeneity, indicating the development of a major process of degeneration. However, there was no change in the plasma level of proaccelerin (factor V), which is also synthesized by the liver, and it seems more likely, therefore, that ochratoxin A intervened at an earlier stage, with a more specific effect on fibrinogen biosynthesis. A complementary study would have to be undertaken for further consideration of this possibility.

The second hypothesis, of disseminated intravascu-

Table 1. *Time course of changes in factors concerned with haemostasis, in bilirubin levels and in haematological parameters in rats treated with 4 mg ochratoxin A/kg/day for 4–10 days*

Parameter	Control values‡	Values† after a treatment period (days) of 4	6	8	10	Significance‡
Total dose administered (mg/kg)...	0	16	24	32	40	
Fibrinogen (g/litre)	2·52 ± 0.19	2·84 ± 0·31	1·53 ± 0·29	0·99 ± 0·25	0·33 ± 0·25	**
Proaccelerin (%)	90·8 ± 3·1	93·8 ± 5·1	94·2 ± 4·7	90·3 ± 4·0	82·9 ± 4·0	
Proconvertin-Stuart factor (%)	87·4 ± 3·7	83·9 ± 6·0	76·3 ± 5·6	57·5 ± 4·7	38·7 ± 4·7	**
Prothrombin (%)	88·9 ± 4·0	93·1 ± 6·6	87·8 ± 6·2	77·0 ± 5·2	60·0 ± 5·2	*
Thrombocytes (1000/mm^3)	781 ± 49	823 ± 81	530 ± 75	532 ± 64	336 ± 64	**
Mean thrombocyte volume (μm^3)	3·33 ± 0·26	3·60 ± 0·14	3·84 ± 0·40	3·93 ± 0·34	4·49 ± 0·38	
Megakaryocytes (no./mm^3)	51·0 ± 7·8	25·2 ± 5·6	12·4 ± 4·3	19·6 ± 6·1	14·2 ± 3·6	**
Bilirubin (mg/litre)	5·61 ± 1·35	9·36 ± 1·86	12·78 ± 1·86	18·96 ± 1·47	15·98 ± 1·42	**
Packed cell volume (%)	33·34 ± 0·70	34·63 ± 0·97	33·48 ± 1·00	38·18 ± 0·76	41·03 ± 0·74	**
Haemoglobin (g/litre)	1·24 ± 0·26	1·31 ± 0·36	1·26 ± 0·32	1·45 ± 0·29	1·53 ± 0·28	**
Erythrocytes (1000/mm^3)	6264 ± 143	6653 ± 198	6596 ± 198	7398 ± 155	7959 ± 151	**
Leucocytes (no./mm^3)	11,304 ± 1060	12,419 ± 1468	12,919 ± 1436	16,138 ± 1155	19,260 ± 1120	**
Neutrophils (no./mm^3)	2708 ± 919	4066 ± 1273	5281 ± 1073	7759 ± 1002	10,133 ± 971	**
Eosinophils (no./mm^3)	81 ± 27	49 ± 37	44 ± 37	59 ± 29	117 ± 28	
Lymphocytes (no./mm^3)	8053 ± 471	8029 ± 653	6594 ± 653	7880 ± 514	8673 ± 498	
Monocytes (no./mm^3)	376 ± 41	360 ± 57	317 ± 57	438 ± 45	344 ± 43	

† Values are means ±SD for groups of 14 ochratoxin-treated rats and 28 controls, the latter being the combined means for seven control animals at each of the four sampling times.

‡ Asterisks indicate those parameters which showed a significant difference (analysis of variance) between the treated animals and the controls: * $P < 0·05$; ** $P < 0·01$.

lar coagulation, is supported by the findings of hypofibrinogenaemia and a negative correlation between thrombocytopenia and the increase in thrombocyte volume. On the other hand we were unable to find any fibrin deposits in the kidneys or digestive tract, organs which are known to be the sites of such coagulation (Boddaert, Amouroux & Roujeau, 1971). However, these formations are only transient, so the 2-day interval between our observations was perhaps too long. On the other hand, the proaccelerin level in the plasma remained unchanged, although it is usually lowered during the clotting process. This hypothesis therefore seems to be doubtful. Unfortunately, the search for fibrinogen degradation products had to be abandoned because the findings obtained in the rat were not interpretable.

Bone-marrow studies indicated the gradual development of a specific megakaryocyte hypoplasia, since red and white blood cell lines were unaltered; indeed erythrocyte and leucocyte counts increased in contrast to the fall in the thrombocyte count, probably because of haemoconcentration and the increase in leucocytes. Haemoconcentration was indicated by the correlation between the increases in packed cell volume, haemoglobin concentration and erythrocyte count, and probably reflected the dehydration characteristic of ochratoxicosis and attributable to the concomitant polyuria and diarrhoea (Hatey & Galtier, 1977). The increase in the leucocyte counts was likely to be partly due to haemoconcentration but particularly to the increase in neutrophils. The aetiology of this observation is difficult to determine, but may be related to the necrotic process that occurs in numerous organs during ochratoxicosis. The thrombocytopenia must therefore be attributed to a decrease in production. The slight increase in thrombocyte volume would be due to a maturation failure associated with a developmental anomaly of the demarcation membranes as occurs in human cases of malignant haemopathia (Paulus, 1975).

In the prothrombin complex, in contrast to the data published by Doerr *et al.* (1976), factor V was unaffected whereas factors II and VII + X showed a slight and gradual decrease. This condition may have stemmed from a decrease in the intake or absorption of vitamin K or from the coumarin-like effect of ochratoxin A. This last possibility is doubtful because the decreases in the levels of the clotting factors were only slight and because there was a 7–8-day delay before the appearance of these changes. Montigel & Pulver (1955) did in fact obtain a similar profile in the rabbit but with a fall in prothrombin and proconvertin levels of some 80% after only 4 days of daily administration of Sintrom, a 4-hydroxycoumarin derivative, in a dose of 1–5 mg/kg.

The appearance of these anomalies could be explained, however, by the decrease in food consumption brought about by the treatment the rats received (Hatey & Galtier, 1977) and to the subsequent diarrhoea. Such diarrhoea interferes with the caecal and intestinal microflora that ordinarily produce endogenous vitamin K and also decreases coprophagy, which is an important source of this vitamin in the rat (Mameesh & Johnson, 1959).

As already mentioned, the changes in fibrinogen and thrombocyte counts preceded by several days of treatment the variations in blood parameters. Since plasma fibrinogen concentration and thrombocyte count are two important factors in haemostasis, this function would be affected before the haematological disturbance became apparent. Thus the dehydration and necrotic process may develop after the more specific disorders which are really responsible for the already reported haemorrhagic syndrome induced by the mycotoxin and for the irreversible process that leads to death of the treated animals.

The haemorrhagic syndrome that appears in the last stages of experimental ochratoxicosis in the rat may be attributed to an extensive hypofibrinogenaemia. This disturbance is due to a defect in hepatic synthesis accentuated by the considerable length of time that ochratoxin A stays in the tissues (Galtier, 1974). The toxic effect is also related to a reduction in the numbers of megakaryocytes. The combination of these two effects leads to the development of a syndrome similar to that of disseminated intravascular coagulation. However, the constant level of proaccelerin in the plasma and the apparent absence of fibrin deposits in the kidney, lung and digestive tract seems to counter this hypothesis. Similarly, a decrease in vitamin K intake could confer an indirect coumarin-like action on ochratoxin A. Lastly, it is interesting to note that a fibrinolytic protease (Kloecking & Markwardt, 1971a) and a thrombolytic protein (Kloecking & Markwardt, 1971b) are synthesized by *Aspergillus ochraceus*, since this fungus can also produce ochratoxin A. Hypofibrinogenaemia and thrombocytopenia could therefore act synergistically under the influence of the mycotoxin, if the toxin, the protease and the thrombolytic protein were all found to contaminate the same foodstuffs.

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PRODUCTION OF [^{3}H]PATULIN OF HIGH SPECIFIC ACTIVITY

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Abstract—In resuspension experiments with *Penicillium patulum*, patulin production was not directly proportional to the glucose concentration of the broth. Proportionally more patulin was produced in broth of low glucose concentration. Patulin production was inversely related to the nitrogen concentration of the resuspension medium. Recovery of tritium as [^{3}H]patulin ranged from 0·15 to 0·30% when ^{3}H-labelled sodium acetate substrate was used. The low tritium recovery is likely to have been due primarily to keto–enol tautomerism of patulin precursors and hydrogen exchange with water. Maximum specific activity of isolated [^{3}H]patulin was 2478 dpm/ng. This specific activity is more than sufficient to make possible *in vivo* experiments on patulin distribution and metabolism.

INTRODUCTION

Patulin, a non-nitrogenous mycotoxin, is occasionally found in human foodstuffs including apple cider (Ware, Thorpe & Pohland, 1974; Wilson & Nuovo, 1973), plums, peaches, pears, apricots and sweet cherries (Buchanan, Sommer, Fortlage, Maxie, Mitchell & Hsieh, 1974). Additionally, there is concern over potential loss to livestock when their feeds are contaminated with patulin (Lovett, 1972; Ukai, Yamamoto & Yamamoto, 1954).

In mammalian acute lethality tests, patulin produced extensive subcutaneous and pulmonary oedema (Broom, Bulbring, Chapman, Hampton, Thomson, Ungar, Wien & Woolfe, 1944; Katzman, Hays, Cain, Van Wyk, Reithel, Thayer, Doisey, Gaby, Carroll, Muir, Jones & Wade, 1944), as well as haemorrhage in the lungs and kidneys, and hepatic congestion (Broom *et al.* 1944). In contrast to the well-known aflatoxins, little is known about *in vivo* distribution and metabolism of patulin, or the mechanism(s) by which it produces its toxic effects, although theories about the latter have been suggested (Ashoor & Chu, 1973).

A probable cause for this paucity of information is the lack of radioactively-labelled patulin of high specific activity, which is essential for appropriate studies. Radioactively-labelled patulin of high specific activity has now been produced, as described below.

EXPERIMENTAL

A desiccated sample of *Penicillium patulum* NRRL 2159A (white mutant) obtained from the USDA Agricultural Research Service, Northern Regional Research Laboratory, Peoria, IL, was cultured on Czapek Solution Agar (Difco Laboratories, Detroit, MI). Spores from this culture were suspended in sterile distilled water and subsequently used to inoculate broth cultures.

Czapek-Dox medium containing 0·3 mg $ZnSO_4 \cdot 7H_2O$/litre was prepared according to the procedure of Scott & Breadling (1974). Cultures used in radioactive resuspension experiments were prepared by the following method. A 500-ml flask containing 125 ml sterile Czapek-Dox medium was inoculated with $2{\cdot}6 \times 10^6$ *P. patulum* spores and shaken on a New Brunswick Co. Model R76 reciprocal waterbath-shaker (New Brunswick, NJ) at 25°C and 170 cycles/min. On day 4 after inoculation, the broth was analysed to ensure that patulin production was occurring and then, on day 4 or 5 after inoculation, mycelial growth in the form of small rice-shaped pellets was removed from the broth and washed with copious sterile distilled water. Excess water was pressed from the pellets with a sterilized spatula, and 1 g of the pellets was added to a previously prepared 50-ml flask.

The 50-ml flask was prepared by first sterilizing it with dry heat at 180°C for 2 hr. An ethanol solution of sodium [^{3}H]acetate was added to the flask and the ethanol was removed by evaporation. Finally, 10 ml of nitrate-free Czapek-Dox broth containing 10 mg glucose was added to the flask before the addition of mycelial pellets. After addition of the pellets, the flask was shaken in a 25°C waterbath at 170 cycles/min until the patulin was harvested. Tritiated sodium acetate for resuspension experiments was obtained from ICN Pharmaceuticals, Inc., Irvine, CA, in two lots at stated activities of 0·8 and 6·0 Ci/mmol.

Radioactive patulin was removed from broth by triple extractions with ethyl ether, the volume of the ethereal phase being twice that of the broth for each extraction. After drying over sodium sulphate, the ether was evaporated, and the residue was dissolved in 0·25 or 0·5 ml chloroform. The total volume of chloroform was spotted on 0·5 mm thick plates of Silica Gel HF 254 (EM Laboratories, Inc., Elmsford, NY) and developed in one of the following solvent systems: toluene–ethyl acetate–formic acid (6:3:1, by vol.), chloroform–glacial acetic acid (9:1, v/v), chloroform–benzene (9:1, v/v), or benzene–methanol (9:1, v/v). Plates were viewed under ultraviolet light, and silica gel at the R_F of the standard was removed from the plate and eluted with absolute alcohol, ethyl acetate or ether. After reduction of the solvent volume,

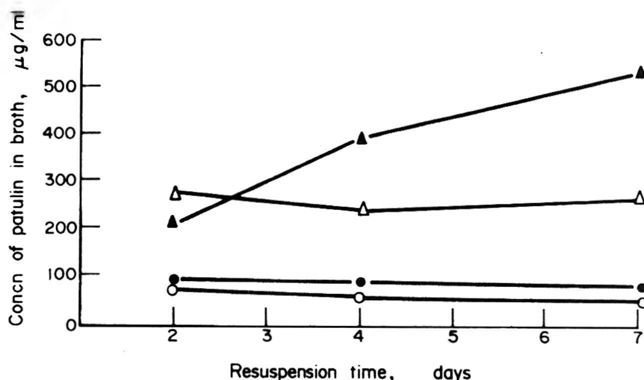

Fig. 1. Patulin production by *P. patulum* in modified Czapek-Dox medium at glucose concentrations of 40 (▲), 10 (△), 3 (●) and 1 (○) g/litre.

the [^{3}H]patulin was again chromatographed in another of the solvent systems just described. Finally, after chromatography in two or more solvent systems, the patulin was eluted from the silica gel, dissolved in absolute alcohol or dichloromethane and scanned spectrophotometrically for quantitation and as an additional check on purity.

For determination of patulin specific activity, 10 μl aliquots of the [^{3}H]patulin solution were combined with 10 ml volumes of Aquasol (New England Nuclear, Boston, MA) in polyethylene vials (New England Nuclear), and counted to an error of 3% or less in a Beckman LS-230 liquid scintillation counter.

Preliminary resuspension experiments with non-radioactive medium were similar to those using [^{3}H]acetate except that the flasks were not shaken, and at resuspension, the entire vegetative contents of the flasks after rinsing were resuspended in 125 ml of modified Czapek-Dox broth at several glucose or nitrate levels.

Crystalline patulin used to prepare standards was produced and purified according to the method of Norstadt & McCalla (1969).

RESULTS

P. patulum resuspension cultures are capable of patulin production at low glucose concentrations, but peak patulin concentrations occur earlier when substrate is limited (Fig. 1). It is important to note that patulin production was not directly proportional to the glucose concentration of the broth. At lower glucose concentrations, proportionally more patulin production occurred than at higher glucose concentrations.

The effect of the nitrogen concentration of the resuspension medium upon patulin production is shown in Fig. 2. A distinct trend is obvious in which patulin concentration of the broth appears to be inversely related to nitrogen content. This effect has been noted by others (Norstadt & McCalla, 1971; Tanenbaum & Bassett, 1959).

In the first attempt to produce tritiated patulin, 5 μmol [^{3}H]acetate (3·96 mCi) was used as substrate as well as 10 mg glucose. This produced a glucose-to-acetate molar ratio of 11:1.

Patulin production at 1, 6, 9 and 13 days resuspension was 29·0, 232, 62·5 and 16·5 μg/ml broth. Specific activity of patulin isolated at these times was 106, 82, 43 and 64 dpm/ng. The four extractions yielded a total of 406 μg [^{3}H]patulin which contained 11·8 μCi tritium. Only 0·30% of the radioactivity added to the flask was recovered as [^{3}H]patulin.

In a later resuspension experiment, 3·47 mCi

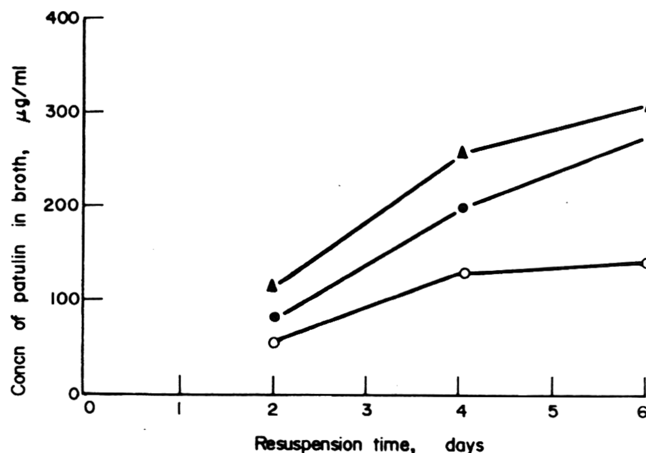

Fig. 2. Patulin production by *P. patulum* in modified Czapek-Dox medium (10 g glucose/litre) at nitrogen concentrations of 0 (▲), 0·5 (●) and 3·0 (○) g $NaNO_3$/litre.

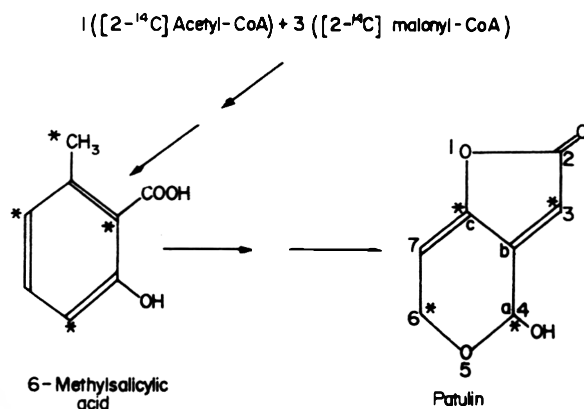

Fig. 3. Biosynthesis of patulin and sites of potential radioactivity (*).

^{3}H-labelled sodium acetate (4·375 μmol) was used. After 2 days of resuspension, 1 ml of the broth was extracted but the ultraviolet spectrum of the TLC-purified patulin gave evidence of contamination which was not removed by further chromatography.

The remainder of the broth was extracted after 3 days of resuspension. The yield was 305 μg [^{3}H]patulin. After several days at room temperature, the ethanol solution of [^{3}H]patulin showed upon spectrophotometric examination an intense absorption peak in the range of 200–225 nm; this was not initially present. The ^{3}H-labelled patulin was again purified by TLC and the specific activity was determined to be 48 dpm/ng. Of the radioactivity added to the flask, 0·20% was recovered in the 305 μg [^{3}H]patulin.

In the final resuspension experiment, 20 ml of a new lot of ^{3}H-labelled sodium acetate was used. The stated activity of this ethanol solution was 6·0 Ci/mmol in a total volume of 25 ml. The first extraction performed after 3·5 days of resuspension yielded only trace amounts of patulin from 1 ml broth. A second extraction after 5·5 days of resuspension produced no detectable patulin.

The pH of the resuspension broth was measured and found to be 8·0, a level incompatible with patulin accumulation (Forrester & Gaucher, 1972; Norstadt & McCalla, 1971). A sterile unused portion of the resuspension broth had a pH of 4·0, so the failure to produce patulin was not due to an error in preparation of the broth.

The pH of the remaining non-extracted resuspension broth was lowered to 5·4 with sterile 0·1 N-HCl, and a sterile solution of 5 mg glucose in 0·25 ml water was added to the culture. After 1 day of shaking, 43·5 μg [^{3}H]patulin was isolated from 0·6 ml of the broth. The remaining broth was then extracted and 595 μg of purified [^{3}H]patulin was obtained. Specific activities of 2414 dpm/ng and 2478 dpm/ng were obtained from the third and fourth extractions respectively.

The cause of the tremendous increase in specific activity of the [^{3}H]patulin over that obtained in previous attempts was discovered when the ethanol solution of sodium [^{3}H]acetate was examined. The activity of this solution was about 24 times greater than that stated by the manufacturer. The 20 mCi of sodium [^{3}H]acetate thought to have been used in the resuspension experiment was approximately 480 mCi. Since 0·713 mCi was recovered as [^{3}H]patulin in this experiment, this corresponded to a 0·15% recovery of the tritium.

This second lot of sodium [^{3}H]acetate in ethanol was analysed for acetate content by the method of Tjan & Jansen (1971), to determine whether the high radioactivity of the solution was primarily due to a higher-than-stated specific activity of the acetate, or to the presence of more [^{3}H]acetate. Approximately 39 times as much acetate was found as was stated by the manufacturer. This extra acetate explains the initial high pH of the resuspension medium and its failure to produce patulin.

DISCUSSION

The apparent decomposition of patulin in ethanol was unexpected. Katzman *et al.* (1944) reported that an alcoholic solution of patulin was stable for 3 months at room temperature in daylight. Tanenbaum & Bassett (1958) found that patulin was converted to "pre-patulin", which has an absorption maximum at 280 nm with a low molar extinction coefficient, after standing for several days in 95% ethanol. The molar absorptivity of patulin in methanol at 277 nm is also known to decrease over a period of days or months (Pohland & Allen, 1970).

The specific activity of the radioactive patulin produced was much greater than that reported by other investigators. Bassett & Tanenbaum (1960), using a substrate of ^{14}C-labelled 6-methylsalicylic acid, obtained patulin with a specific activity of 3960 cpm/μmol. Forrester & Gaucher (1972) used sodium [1-^{14}C]acetate substrate to obtain patulin with a specific activity of $4·4 \times 10^4$ dpm/mg. Tanenbaum & Bassett (1959) reported production of patulin with a specific activity of 18,400 cpm/μM using sodium [2-^{14}C]acetate substrate.

The cause for the high specific activity obtained is the relatively large amount of radioactivity (3·47 to 480 mCi) used in the resuspension broths. Also, glucose concentrations were purposely kept low so that the [^{3}H]acetate/glucose ratios would be high. Hsieh & Mateles (1971) have shown that the specific activity of aflatoxins produced by an Aspergillus mould is dependent upon the glucose/acetate molar

ratio when ^{14}C-labelled acetate is used as a substrate. High glucose concentration promoted more biosynthetic activity, resulting in the production of more aflatoxin, and a greater total incorporation of radioactivity into aflatoxin. If, however, the glucose concentration were low, there would be less aflatoxin produced, and less radioactivity recovered as aflatoxin, but the specific activity of the aflatoxin produced would be higher.

Although the specific activity of the ^{3}H-labelled patulin produced by the resuspension experiments may be higher than those reported elsewhere, it is quite low when one considers the amount of radioactivity added to the resuspension broths. Indeed, only 0·15 to 0·30% of the radioactivity added to the resuspension broth was recovered as [^{3}H]patulin in the various experiments. It is likely that there are several reasons for this low recovery of radioactivity.

The first aromatic precursor of patulin, 6-methylsalicylic acid, is formed by condensation of acetyl- and malonyl-CoA. According to the biosynthetic scheme for 6-methylsalicylic acid subscribed to by Scott, Breadling, Georgopapadakou & Subbarayan (1974), one would predict that the sodium acetate C-2 carbons, which bear the ^{3}H label, could end up in the 1, 3 and 5 positions of the aromatic ring, as well as in the methyl group of 6-methylsalicylic acid. If this acid is converted to patulin by one or more of the pathways proposed by Scott, Zamir, Phillips & Yalpani (1973), the C-2 carbons of the acetate would be present in the four positions of patulin indicated in Fig. 3. Upon degradation of patulin synthesized from sodium [2-^{14}C]acetate by *P. patulum*, Tanenbaum & Bassett (1959) found the radioactivity to be present in these locations. Thus ^{3}H potentially could be attached to any or all of these four carbons.

Deuterated *m*-cresol derived from 6-methylsalicylic acid loses a deuterium atom at the number 4 position when it undergoes *p*-hydroxylation (Scott *et al.* 1973). Also, the hydrogen on the methyl group of *m*-cresol is not incorporated into patulin (Scott *et al.* 1973). This means that patulin synthesized from ^{3}H-labelled sodium acetate can have tritium labels only on carbons 3 and 6. This loss of tritium from aromatic patulin precursors partially explains the low recovery of tritium in [^{3}H]patulin.

Forrester & Gaucher (1972) used the tritiated patulin precursors, *m*-cresol, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde and gentisaldehyde in fermentor-culture experiments to produce [^{3}H]patulin. Incorporation of substrate activity into patulin ranged from 16·5 to 41%. This suggests that the major loss of tritium during the synthesis of patulin from acetate occurs during the synthesis of 6-methylsalicylic acid.

In the synthesis of malonyl-CoA from acetyl-CoA and CO_2, a hydrogen atom is lost from the carbon-2 of the acetyl-CoA.. Since three malonyl-CoA molecules are used in the synthesis of 6-methylsalicylic acid, numerous opportunities exist for tritium loss in the synthesis of malonyl-CoA. A second and possibly much more important way for tritium loss to occur is through keto–enol tautomerism.

In the first condensation of acetate and malonate during the synthesis of 6-methylsalicylic acid, acetoacetyl-enzyme is formed. This compound contains two carbonyl functions separated by a single methylenic carbon, so extensive tautomerism would be expected to occur. The methylenic carbon in this case is also the number 2 carbon of sodium acetate. Since the C-2 carbon of ^{3}H-labelled sodium acetate is the one containing the tritium label, it is easy to see how loss of tritium can occur through tautomerism and hydrogen exchange with water. All the other non-aromatic precursors of 6-methylsalicylic acid between acetoacetylenzyme and the product contain multiple ketonic functions in which ^{3}H loss through tautomerism would be expected to occur.

Although the percentage incorporation of tritium from ^{3}H-labelled acetate into patulin was low, the relatively low expense of [^{3}H]acetate in combination with the high specific activities available will permit production of sufficient [^{3}H]patulin of a specific activity of 100 dpm/ng or greater to allow *in vivo* experiments on the distribution and metabolism of patulin.

If appropriate antibodies were available, [^{3}H]patulin could be produced with high enough specific activity to develop a radioimmunoassay for patulin at a reasonable cost.

Such patulin would also be useful for *in vivo* investigations on the mechanism(s) of patulin toxicity such as its supposed alkylation of sulphydryl-containing compounds (Ashoor & Chu, 1973).

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THE TOXICITY OF α-TOMATINE TO *TETRAHYMENA PYRIFORMIS**

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Abstract—The toxic effect of α-tomatine at the molecular level was studied using *Tetrahymena pyriformis* as a model cell system. α-Tomatine at 16·5 ± 4·4 ppm inhibited cell growth by 50%. Cells exposed to α-tomatine increased in volume, and when concentrations were greater than 37·5 ppm, cell lysis started. In addition, α-tomatine inhibited the synthesis of DNA and RNA while stimulating the synthesis of proteins. A concentration of 12·5 ppm α-tomatine inhibited the synthesis of lipids and glycogen, but higher concentrations of the test compound in the medium stimulated the synthesis of glycogen. α-Tomatine did not affect the oxidation of Na [1-^{14}C]acetate to labelled CO_2. Exposure to the alkaloid for 24 hr altered the percentage of radioactive label incorporated into lipids by decreasing the synthesis of phospholipid and increasing the synthesis of monoglycerides, diglycerides, triglycerides, tetrahymenol and squalene. α-Tomatine also altered the proportions of polar lipids synthesized. The mode of toxicity is attributed to a perturbation of the cell membrane by the alkaloid.

INTRODUCTION

α-Tomatine [(22S, 26R), 5α-tomatin-3β-(1→3)-β-D-glucopyranosyl-(1→2)-*O*-β-D-(3-*O*-β-D-xylopyranosyl-glucopyranosyl)-(1→4)-*O*-β-D-galactopyranoside] (Fig. 1) is a glycoalkaloid shich has been isolated from tomatoes. The chemical and biochemical aspects of this compound were reviewed recently by Roddick (1975).

The biological activity of α-tomatine is similar to that of α-solanine and α-chaconine, the alkaloid-saponins found in potatoes (Nishie, Norred & Swain, 1975). The ip LD_{50} in mice for α-tomatine is 33·5 mg/kg body weight compared with 30·0 mg/kg body weight for α-solanine and 27·5 mg/kg for α-chaconine. In contrast to the potato alkaloids, α-tomatine has a low oral toxicity of 500 mg/kg body weight. The difference between the oral LD_{50} and ip LD_{50} is attributed to the poor absorption of α-tomatine from the gastro-intestinal tract. α-Tomatine exerts a positive inotropic effect on cardiac contractions in the frog and this effect is greater than that of either α-solanine or α-chaconine (Nishie, Fitzpatrick, Swain & Keyl, 1976). In addition, α-tomatine haemolyses erythrocytes both *in vivo* and *in vitro* (Wilson, Poley & DeEds, 1961) with 50% haemolysis occurring at 8 ppm (Segal & Schlosser, 1975).

Tetrahymena pyriformis, a ciliated protozoan, has nutritional requirements, subcellular organelles and biochemical pathways similar to those of mammalian cells (Hill, 1972). The cell is a useful model for the study of dynamic membrane alterations since the system has been well studied, has a typical eukaryotic membrane, and responds quickly to a wide variety of physical and chemical stimulants by altering membrane metabolism (Thompson & Nozawa, 1977). In addition, *T. pyriformis* synthesizes most lipids and proteins for internal use, so the compounds can be traced throughout the cellular lifetime (Thompson & Nozawa, 1977). This organism has been shown to be a useful model for studying the mode of action, at the cellular level, of food additives and pesticides (Rankin, Surak & Thompson, 1977; Surak, 1977; Surak, Bradley, Branen, Ribelin & Shrago, 1976a; Surak, Bradley, Branen & Shrago, 1976b). This report describes the mode of action of α-tomatine at the cellular level.

EXPERIMENTAL

Materials. α-Tomatine was obtained from Sigma Chemical Co., St. Louis, MO, crystalline bovine serum albumin from Calbiochem Co., San Diego, CA, proteose peptone and yeast extract from Difco Laboratories, Detroit, MI, [*Me*-^{3}H]thymidine, [5-^{3}H]uridine and Na [2-^{14}C]acetate from New England Nuclear, Boston, MA, and Soluene 350 from Packard Instrument Co., Downers Grove, IL. All other chemicals were reagent grade.

[^{14}C]Tetrahymenol was prepared by the addition of 2 μCi, [2-^{14}C]acetate to 200 ml culture of *T. pyriformis* in early log-phase growth, allowing the culture to incubate for 48 hr before isolating and purifying the compound according to a modified procedure of Thompson, Bamberly & Nozawa (1971). Phospholipids were removed from the crude lipid extract by

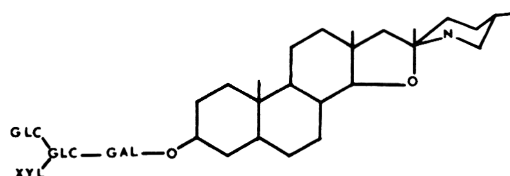

Fig. 1. The structure of α-tomatine: GAL, β-D-galactopyranosyl group; GLC, β-D-glycopyranosyl group; XYL, β-D-xylopyranosyl group.

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passing it through a silica-gel column in $CHCl_3$. The neutral lipids were subjected to saponification with methanolic-KOH and the tetrahymenol was extracted with benzene. Water was removed from the final benzene extract with anhydrous Na_2SO_4. The purity of the tetrahymenol was assayed on a gas-liquid chromatograph according to the procedure of Thompson *et al.* (1971) and was found to be >99% pure.

To measure cell growth, *T. pyriformis* was incubated without shaking at 23 ± 1° in a sterile medium consisting of 2% proteose peptone and 0·1% yeast extract. For experimental purposes of 2% inoculum of early log growth cells was used. α-Tomatine as a concentrated solution dissolved in acetic acid was added to cultures so that the final concentration of α-tomatine ranged from 0 to 50 ppm and the final concentration of acetic acid was 0·45 mM. Cell numbers and size distribution were measured using a Coulter Counter Model ZBI equipped with a 200-μm orifice and previously calibrated with polystyrene beads.

Membrane permeability of *T. pyriformis* was determined by measuring the leakage of cellular proteins in Wagners solution (Rothstein & Blum, 1973), a non-nutritive medium. Early log-growth cultures were washed three times in Wagners solution and resuspended to the original cellular density. After 3 hr of incubation the cells were harvested by centrifugation at 5000 g and the amount of protein in the incubation medium was measured fluorimetrically with an excitation of 288 nm and an emission of 336 nm (Resch, Imm, Feber, Wallach & Fisher, 1977). Crystalline bovine serum albumin was used as a standard.

Radioactive measurements. The procedures of Shug, Elson & Shrago (1969) and Milner (1967) as modified by Surak *et al.* (1976b) were used to determine the effect of α-tomatine on the incorporation of Me-[^{3}H]-thymidine into RNA, [5-^{3}H]uridine into DNA, and Na[1-^{14}C]acetate or Na-[2-^{14}C]acetate into lipids, glycogen and protein. The major lipid classes were separated by thin-layer chromatography, TLC, on a silica gel G plate using hexane–diethyl ether–acetic acid (85:15:2, by vol.) as the mobile solvent, and polar lipids were separated by TLC on a silica gel G plate using chloroform–acetic acid–methanol–water (74:25:5:2·2, by vol.) (Nozawa, Fukushima & Iida, 1973). Compounds were identified by comparing R_F values of isolated lipids with authentic lipid standards. The TLC plates were scraped into scintillation vials containing an appropriate toluene-based scintillation fluid and the radioactivity was counted in a liquid scintillation counter. Quenching was corrected using the automatic external standardization device of the counter. The procedure of Saba & DiLuzio (1966) as modified by Surak (1977) was used to determine the effect of α-tomatine on the tricarboxylic acid cycle by measuring the oxidation of Na [1-^{14}C]acetate to labelled CO_2.

The formation of a complex between α-tomatine and [^{14}C]tetrahymenol was measured by mixing equal amounts of 1·0 mM α-tomatine dissolved in 95% ethanol and 1·0 mM [^{14}C]tetrahymenol dissolved in acetone–diethyl ether (1:1, v/v). After 16 hr the complex was centrifuged, washed once with acetone and dissolved in Soluene 350. The radioactivity in the α-tomatine–tetrahymenol precipitate was measured along with an aliquot of the supernatant in a scintillation counter with an appropriate toluene-based cocktail. Quenching was corrected by automatic external standardization.

Experiments were performed at least three times and data presented are means ±SEM of a triplicate analysis of a single experiment.

RESULTS

Growth and morphology

Increasing concentrations of α-tomatine inhibited the growth of early log growth phase cultures of *T. pyriformis* (Fig. 2). After 24 hr of exposure, there was a dose-dependent decrease in cell numbers when the concentration of α-tomatine was greater than 6·25 ppm (Fig. 3). The effective dose at which 50% growth inhibition occurred (ED_{50}) was 16·5 ± 4·4 ppm.

A 50% increase in cell volume was observed when cultures were exposed for 3 hr to 37·5 ppm α-tomatine. *T. pyriformis* exposed for 24 hr to concentrations greater than 25·0 ppm of the alkaloid had a mean cell volume of 4595 μm^3 as compared to 3065 μm^3 for the control cultures (Fig. 4). Viable treated cells exhibited normal mobility and normal gross morphology. However, when the concentration of α-tomatine was 37·5 ppm, some cells appeared to have swelled and lysed. At concentrations greater than 50 ppm no viable cells were observed.

The effect of α-tomatine on membrane permeability was determined by measuring the leakage of cellular proteins into a non-nutritive medium after a 3-hr incubation. At concentrations of α-tomatine less than 15 ppm, there was no increase in leakage of proteins from the cells (Fig. 5). When the concentration of

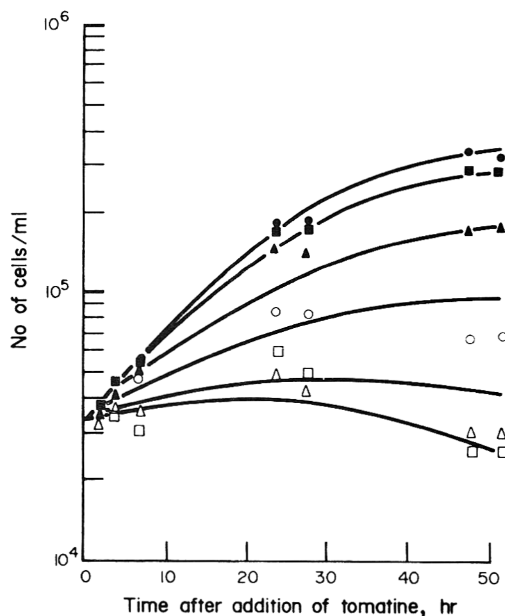

Fig. 2. Growth curves of *T. pyriformis* after addition of α-tomatine as a concentrated solution in 0·45 mM acetic acid to an early log-growth culture. Concentrations of α-tomatine in the medium were 0 (●), 6·25 (■), 12·5 (▲), 25 (○), 30 (□), 37·5 (△) ppm.

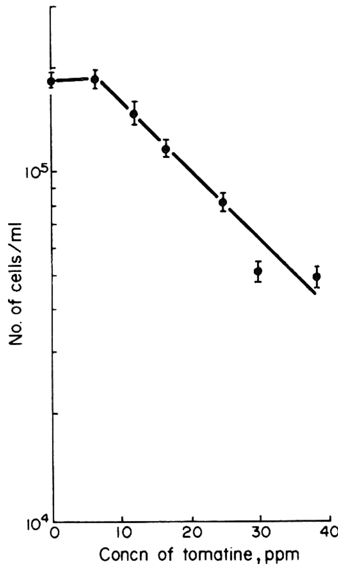

Fig. 3. Cell concentration of *T. pyriformis* after incubation for 24 hr with α-tomatine added as a concentrated solution in 0·45 mM acetic acid to an early log-growth culture.

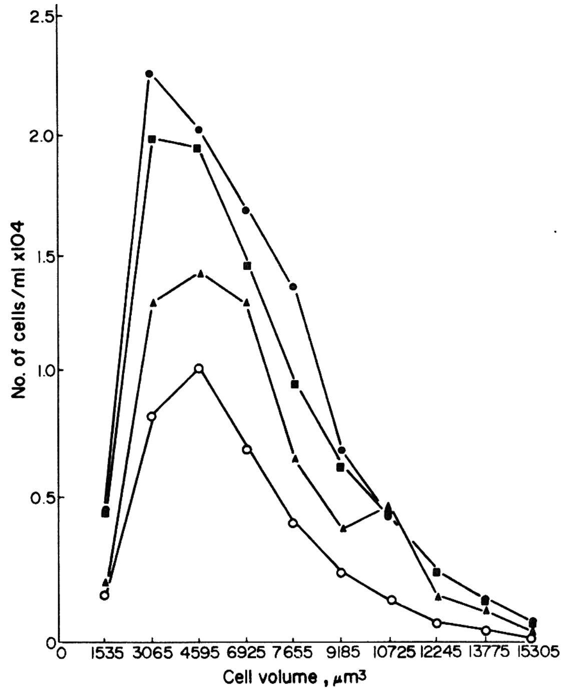

Fig. 4. Size distribution of *T. pyriformis* after incubation for 24 hr with α-tomatine added as a concentrated solution in 0·45 mM acetic acid. Concentrations of α-tomatine in the medium were: 0 (●), 12·5 (■), 25 (▲), 37·5 (○) ppm.

α-tomatine was greater than 15 ppm, there was an increase in the amount of cellular proteins in the medium which was proportional to the decrease in cell number caused by lysis (Fig. 5).

Radioisotope measurements

α-Tomatine stimulated the synthesis of proteins from [^{14}C]-labelled amino acids while depressing the synthesis of both DNA and RNA (Table 1). The alkaloid inhibited glycogen synthesis and lipid synthesis at 12·5 ppm; however, increasing concentrations of α-tomatine in the medium stimulated glycogen synthesis and had only a slight effect on lipid synthesis (Table 1). When the α-tomatine concentration was 37·5 ppm, lipid synthesis was 2% less than the control and the glycogen synthesis was 50% greater than the control. During the observation period the compound had no significant effect on the oxidation of [^{14}C]acetate to $^{14}CO_2$ (Table 1).

The percentage of [^{14}C]acetate incorporated into the major lipid classes and polar lipids was measured to determine whether the compound would alter lipid metabolism. After the cultures were exposed for 3 hr

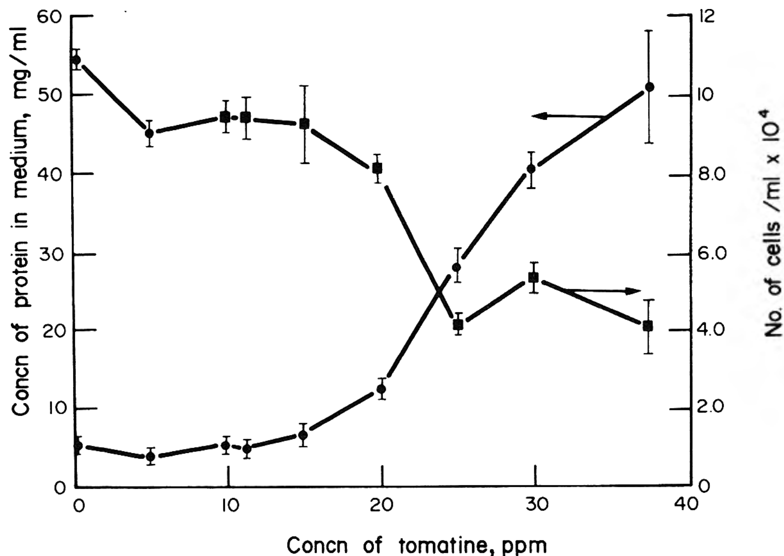

Fig. 5. Effects of various concentrations of α-tomatine on membrane permeability of *T. pyriformis* (●) and cell number (■). Membrane permeability was measured by quantifying the leakage of cellular proteins into a non-nutritive medium.

Table 1. α-Tomatine effect on the synthesis of macromolecules, lipids and CO_2

Concn of α-tomatine (ppm)	Radioactivity (dpm/10^5 cells) incorporated in					
	DNA*	RNA†	Protein‡	Lipid‡	Glycogen‡	CO_2‡
0	11.015 ± 425	67.749 ± 12.361	13.670 ± 2521	9635 ± 1095	945 ± 228	313,628 ± 16,070
12·5	10.988 ± 933	54.672 ± 11.086	14.538 ± 908	6913 ± 826	719 ± 134	313.568 ± 12.030
25·0	8113 ± 733	49.865 ± 18.382	17.099 ± 1748	8813 ± 63	1020 ± 155	270.494 ± 22.950
37·5	7823 ± 126	46.983 ± 14.379	17.729 ± 1371	9324 ± 99	1426 ± 85	318.906 ± 18.497

*Synthesis determined using [^{3}H]thymidine.
†Synthesis determined using [^{3}H]uridine.
‡Synthesis determined using Na [1-^{14}C]acetate.
Values are means ±SEM the amount of radioactivity incorporated into various purified fractions after a 3-hr incubation with 1·0 μCi of the appropriate isotope.

to α-tomatine, a decrease in the percentage of phospholipids and an increase in the percentage of triglycerides were observed (Table 2). After 24 hr phospholipid synthesis was depressed, and the synthesis of monoglycerides, diglycerides, triglycerides, tetrahymenol and squalene was increased at all α-tomatine levels (Table 2). Changes in the amount of label being incorporated into different polar lipids were observed after 3 hr exposure (Table 3). The amounts of lysophosphatidylethanolamine, lyso-2-aminoethylphosphonolipid and 2-aminoethylphosphonolipid synthesized increased. After 24 hr of exposure, there were marked changes in the patterns of polar lipids synthesized by treated cells. The data in Table 3 show an increase in the incorporation of [^{14}C]acetate into lysophosphotidylethanolamine, lyso-2-aminoethylphosphonolipid and an unidentified polar lipid which migrated at an R_F intermediate to phosphatidylcholine and phosphatidylethanolamine. A decrease in the synthesis of phosphatidylcholine, phosphatidylethanolamine and 2-aminoethylphosphonolipid was also observed (Table 3).

Equimolar concentrations of α-tomatine and [^{14}C]tetrahymenol were mixed to determine whether the two compounds form an insoluble principle similar to the α-tomatine–cholesterol complex. After 24 hr, a small amount of precipitate was collected in the bottom of the reaction vessel containing 2·97 ± 1·27% of the [^{14}C]tetrahymenol. This compares with the 1:1 complex which is formed when equimolar amounts of α-tomatine and cholesterol are mixed together (Karabara, McLaughlin & Riegel, 1961).

DISCUSSION

T. pyriformis is an excellent dynamic model for studying alterations in membrane metabolism caused by physical or chemical agents since its membranes are equivalent to those of other eukaryotic cells and it has the ability to alter membrane physical properties and composition when faced with sudden perturbations (Thompson & Nozawa, 1977). The cell will alter its membrane composition in order to regulate membrane fluidity. For example, membrane fluidity

Table 2. *Effect of α-tomatine on percentage of Na 2-[^{14}C]acetate incorporated into the major lipid components of* T. pyriformis *when cultures were exposed to the alkaloid for 3 hr or 24 hr*

Lipid class	α-Tomatine (ppm)...	Na[2-^{14}C]acetate incorporated (%) 0	12·5	25·0	37·5
		3-hr incubation*			
Phospholipids		48·78 ± 1·67	47·15 ± 2·15	46·02 ± 0·65	44·03 ± 1·67
Monoglycerides and diglycerides		2·06 ± 0·12	2·16 ± 0·13	2·09 ± 0·07	2·21 ± 0·12
Tetrahymenol		3·52 ± 1·31	3·56 ± 1·37	4·20 ± 0·17	2·86 ± 1·08
Free fatty acids		6·60 ± 0·31	6·10 ± 1·10	5·66 ± 0·28	5·62 ± 1·04
Triglycerides		37·60 ± 2·25	39·62 ± 4·05	40·91 ± 0·58	43·16 ± 1·20
Squalene		0·73 ±	0·87 ± 0·40	0·40 ± 0·02	1·21 ± 0·43
		24-hr incubation†			
Phospholipids		54·99 ± 2·13	31·65 ± 1·72	30·36 ± 0·56	29·74 ± 3·19
Monoglycerides and diglycerides		1·69 ± 0·04	3·87 ± 1·90	3·23 ± 0·18	3·90 ± 1·51
Tetrahymenol		3·60 ± 0·78	5·91 ± 1·39	4·24 ± 0·76	7·78 ± 0·97
Free fatty acids		2·05 ± 0·11	3·62 ± 0·35	4·19 ± 0·02	6·16 ± 0·80
Triglycerides		36·44 ± 2·03	53·00 ± 1·53	54·94 ± 0·57	50·37 ± 3·26
Squalene		0·72 ± 0·78	1·07 ± 0·55	2·23 ± 0·80	1·07 ± 0·32

*α-Tomatine and 1·0 μCi of Na [2-^{14}C]acetate were added together to an early log-growth culture and allowed to incubate for 3 hr prior to the extraction, purification and quantification of the lipids.
†α-Tomatine was added to the culture 24 hr before the extraction and 1·0 μCi of Na [2-^{14}C]acetate was again added 3-hr before extraction, purification and quantification of the neutral lipids.
Values are means ±SEM.

Table 3. *Effect of α-tomatine on the percentage of Na [2-^{14}C]acetate incorporated into various polar lipids of* T. pyriformis *when cultures were exposed to α-tomatine for 3 hr or 24 hr*

Polar lipid class	α-Tomatine (ppm)... 0	12·5	25·0	37·5
	Na [2-^{14}C]acetate incorporated (%)			
3-hr incubation				
Lysophosphatidylcholine	4·54 ± 0·52	4·64 ± 1·10	5·37 ± 1·59	2·56 ± 1·22
Lysophosphatidylethanol-amine and lyso-2-amino-ethylphosphonolipid	2·02 ± 0·08	3·03 ± 0·29	3·77 ± 1·83	3·44 ± 0·64
Phosphatidylcholine	25·60 ± 0·81	26·01 ± 1·01	25·32 ± 0·53	25·50 ± 0·40
Unknown 1	10·40 ± 0·87	9·30 ± 0·32	10·03 ± 0·59	9·27 ± 0·20
Phosphatidylethanolamine	42·28 ± 0·85	42·27 ± 0·80	38·91 ± 4·39	42·34 ± 0·80
2-aminoethylphosphono lipid	12·23 ± 0·19	12·73 ± 0·27	13·74 ± 0·39	13·86 ± 0·22
Unknown 2	1·42 ± 0·14	1·57 ± 0·31	2·09 ± 1·05	1·83 ± 0·30
Cardiolipin	1·51 ± 1·71	0·45 ± 0·35	0·77 ± 0·51	1·20 ± 0·91
24-hr incubation†				
Lysophosphatidylcholine	2·88 ± 0·24	3·22 ± 0·16	2·33 ± 0·46	2·20 ± 1·26
Lysophosphatidylethanol-amine and lyso-2-amino-ethylphosphonolipid	3·00 ± 0·42	4·88 ± 0·22	6·29 ± 0·41	5·02 ± 3·08
Phosphatidylcholine	25·59 ± 0·63	20·88 ± 0·15	16·05 ± 0·55	20·44 ± 4·60
Unknown 1	14·29 ± 0·58	22·54 ± 1.04	28·34 ± 0·64	23·90 ± 2·74
Phosphatidylethanolamine	42·20 ± 0·92	35·45 ± 1·13	34·11 ± 1·06	37·50 ± 2·28
2-aminoethylphosphono lipid	11·01 ± 0·38	10·40 ± 0·22	9·38 ± 0·58	7·73 ± 0·19
Unknown 2	0·41 ± 0·02	0·67 ± 0·07	0·71 ± 0·05	0·70 ± 0·13
Cardiolipin	0·62 ± 0·07	1·95 ± 0·14	2·76 ± 0·22	2·30 ± 0·32

*α-Tomatine and 1·0 μCi of Na [2-^{14}C]acetate were added together to an early log-growth culture and allowed to incubate for 3 hr before extraction, purification and quantification of the lipids.
†α-Tomatine was added to the culture 24 hr before the extraction and 1·0 μCi of Na [2-^{14}C]acetate was again added 3 hr before extraction, purification and quantification of the lipids.
Values are means ±SEM.

is maintained by altering the chain length, by altering degree of unsaturation of fatty acids, by altering the ratio of phospholipids to tetrahymenol (a β-OH-triterpenoid alcohol that has the same membrane function as cholesterol), and by altering the ratio of polar head groups of polar lipids (Conner & Stewart, 1976; Ferguson, Conner & Mallory, 1971; Ferguson, Davis, Conner, Landrey & Mallory, 1975; Fukushima, Martin, Iida, Kitajima, Thompson & Nozawa, 1976; Thompson, Bamberly & Nozawa, 1972).

α-Tomatine has an initial toxic effect on *T. pyriformis* possibly by inserting into the lipid bilayer rather than by binding strongly to tetrahymenol, since α-tomatine forms only a weak complex with tetrahymenol. The organism will alter its membrane composition in response to perturbations of α-tomatine by decreasing the synthesis of phospholipids and by increasing the synthesis of tetrahymenol (Table 2). Since the change in synthetic pathways occurred over a 24-hr exposure to α-tomatine (Table 2), a change in the molar ratio of tetrahymenol to phospholipid would be expected. Chemically-induced alterations of lipid synthesis with respect to membrane metabolism have not been described in the literature. However, changes in membrane fluidity have been observed when ergosterol was added to cultures of *T. pyriformis* (Nozawa, Fukushima & Iida, 1975). Ergosterol replaces tetrahymenol in the membrane on an equimolar basis and inhibits tetrahymenol synthesis. There is a decrease in phospholipid synthesis with a fourfold increase in the molar ratio of steroid to lipid phosphorus (Nozawa *et al.* 1975). In addition, *T. pyriformis* appears to be countering the effect of α-tomatine by altering the proportions of the various lipids synthesized, a development that also leads to an overall change in membrane composition (Table 3). Alterations in the ratio of polar lipids have been observed when membrane fluidity was changed by either the replacement of tetrahymenol with ergosterol (Nozawa *et al.* 1975) or an increase in the incubation temperature (Fukushima *et al.* 1976). The actual significance of changes in the amounts of individual polar lipids is unknown, but the composition of polar lipids is important in regulating the activity of membrane-bound enzymes (Thompson & Nozawa, 1977).

It has been postulated that when *T. pyriformis* membranes are perturbed, the cell will respond first by altering the fatty acid composition and the ratio of tetrahymenol to phospholipid, the change in the phospholipid distribution being a secondary long-term effect (Thompson & Nozawa, 1977). However, the data in Table 3 appear to show that slight alterations in the synthetic patterns of polar lipids occur within 3 hr of exposing the cell to tomatine.

T. pyriformis appears to be able to compensate for higher α-tomatine levels to some extent, since there

is no leakage of cellular proteins in cells exposed to sublytic levels (Fig. 5). However, when concentrations of α-tomatine are greater than 25 ppm, the cell does not completely adapt to the presence of α-tomatine in the membrane since swelling (Fig. 4) and lysis occur. There appears to be some binding of the α-tomatine to the proteose peptone–yeast extract medium since cell lysis was not observed until the concentration of α-tomatine was 37·5 ppm or more. This contrasts with cells exposed to α-tomatine in a non-nutritive medium; lysis was observed in this medium when the concentration was 25 ppm.

The increase in triglyceride synthesis can be attributed to the decrease in phospholipid synthesis and is not necessarily a direct response to a toxic chemical. When *T. pyriformis* is exposed to stimuli that decrease the phospholipid synthesis, the cell does not necessarily decrease the fatty acid production. Borowitz & Blum (1976) demonstrated that triglycerides were not solely produced by the cell for fuel storage and reported an active metabolic link between triacetylglycerol metabolism and phospholipid metabolism. Cells exposed to toxic stimuli may continue fatty acid synthesis and store them in triglycerides for possible further use in phospholipid metabolism.

It has been postulated that α-tomatine will inhibit the oxygen metabolism of *Escherichia coli* (Roddick, 1975). However, this does not appear to be a mechanism of toxicity in the eukaryotic cell. These is no inhibition of the tricarboxylic acid cycle since increasing concentrations of α-tomatine did not inhibit the oxidation of [1-^{14}C]acetate to labelled CO_2 (Table 1). Therefore, there would probably be no inhibition of the electron-transport system.

At the present time the effect of α-tomatine on the synthesis of DNA, RNA and protein cannot be fully explained (Table 1). However, Thompson & Nozawa (1977) reported that a great number of stimuli that alter cell metabolism do so by altering membrane metabolism.

It has been postulated that α-tomatine might have more than one mechanism of toxicity at the cellular level (Arneson & Durbin, 1968). The hydrolytic products of α-tomatine—β_1-tomatine (minus a xylose), β_2-tomatine (minus a glucose) and tomatidine (the aglycon)—do not form a precipitable cholesterol complex, but these compounds have reduced antifungal activity when compared with α-tomatine. In addition, fungi that do not contain steroids (e.g. *Phythium* and *Phytophthora*) are less sensitive to α-tomatine than fungi that contain membrane-bound steroids. This indicates that the sterol binding is a cellular mechanism of toxicity but another mechanism of toxicity may also be present. *T. pyriformis* provides an excellent model system for studying the mode of action of α-tomatine, since it has a dynamic membrane and a strong complexation between the compound and steroids does not occur. Research is continuing to investigate the effects of the action of α-tomatine on other membrane functions using *T. pyriformis.*

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ABSORPTION OF BLANKOPHOR BHC FROM A DETERGENT SOLUTION APPLIED TO THE SKIN OF PIGS

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Abstract—The dermal absorption of BLANKOPHOR BHC, a fluoroscent whitening agent (FWA), was studied in three pigs. A 0·02% solution of [^{14}C]BLANKOPHOR BHC in a 0·5% detergent solution was applied under occlusion to a skin area of approximately 500 cm^2 on each pig for 10 hr. Absorption of tritiated water, determined in parallel on the same animals, showed the rate of water absorption through the skin to be 1·4 mg cm^{-2} hr^{-1}. This is very close to the rate of water absorption determined in human skin, so that the results of BLANKOPHOR BHC absorption obtained on the skin of pigs can be extrapolated to human skin. The maximum rate of absorption of ^{14}C activity, expressed as BLANKOPHOR BHC, was 1·6 ng cm^{-2} hr^{-1}, compared with the 1·4 mg cm^{-2} hr^{-1} for water absorption under the same conditions. Maximum absorption per hour was 0·02% of the dermally applied dose of BLANKOPHOR BHC. At the end of the 10-hr period of exposure under occlusion, the levels of ^{14}C activity in the organs and tissues of the pigs ranged from 0·1 to 1·3 ppb. In the skin, the FWA showed a high affinity for the stratum corneum. Autoradiographic and radiometric studies indicated that delayed absorption of the FWA remaining on or in the skin at the end of the 10-hr period would be negligible or non-existent.

INTRODUCTION

BLANKOPHOR BHC® is a fluorescent whitening agent (FWA). This paper describes studies conducted for the purpose of establishing whether and to what extent it is absorbed through the skin. In view of its practical use in detergents, BLANKOPHOR BHC was dissolved for the tests in an FWA-free detergent. Optimal absorption was achieved by applying the detergent containing BLANKOPHOR BHC for a contact time of 10 hr to approximately 7% of the total skin area of the pig and by covering the treated area with an occlusive dressing. Domestic pigs were used in these studies because their skin displays greater morphological and functional similarity to human skin than is the case with other laboratory animals (Meyer, Schwarz & Neurand, 1977).

To ensure the detection of traces (< 1 ppb) of the absorbed FWA in the tissues and excreta of the treated animals, use was made of BLANKOPHOR BHC radiolabelled with carbon-14 in the triazole rings of the molecule. Therefore, the reported results of the ^{14}C measurements and the autoradiographic studies relate to both the unchanged whitener and its biotransformation products. The amount of water absorbed from the detergent solution under the test conditions was determined by adding tritiated water to the applied solution and measuring the tritium level in the animal tissues.

Besides presenting data on the absorption of the FWA and water from the applied detergent solution during the 10-hr exposure period, the paper discusses the possibility that BLANKOPHOR BHC incorporated into the epidermis might be further absorbed after termination of the 10-hr period.

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EXPERIMENTAL

Materials. BLANKOPHOR BHC labelled with carbon-14 (disodium salt of 4,4′-bis-(4-phenyl-1,2,3-[4-^{14}C]triazol-2-yl)stilbene disulphonate) had a specific activity of 76 μCi/mg, and its radiochemical purity was 91–92% *trans* isomer and 0·5–1% *cis* isomer. The FWA-free detergent was dissolved in completely desalted water with addition of tritiated water to give a concentration of 5 g/litre. The test solution applied dermally to the experimental animals was made up by adding 0·2 mg [^{14}C]BLANKOPHOR BHC/ml to this detergent solution and had a radioactivity of 15 μCi ^{14}C/ml and 105 μCi ^{3}H/ml.

Animals and experimental design. Three male German domestic pigs were bathed in clear water on the morning of the application and an intact area of skin was marked on the flank of each pig. The bristles were removed with hair clippers (0·1 mm) and the clipped area was thoroughly washed with an approximately 1% solution of the FWA-free detergent, rinsed with plenty of clear water and rubbed dry. This skin area of approximately 500 cm^2 (Table 1) was covered with an occlusive dressing, consisting of 50 μm-thick polythene with an incorporated dosing tube and kept in place with Terokal 504 glue (Teroson GmbH, Heidelberg). A 20·0-ml dose of the test solution (4 mg BLANKOPHOR BHC) was applied to the skin through the dosing tube. After removal, by suction, of the cushion of air beneath the polythene dressing, the dosing tube was sealed and the tightness of the dressing was checked. The test solution, which had a mean level of approximately 0·4 mm above the skin surface, was evenly spread over the site of application. The occluded area of skin was wetted with detergent solution throughout the 10-hr contact time. In order to maintain completely satisfactory experi-

Table 1. *Application of [^{14}C]BLANKOPHOR BHC to pigs*

Animal no.	Body weight (kg)	Body surface area* (cm^2)	Area of application site: In cm^2	Area of application site: As % of total skin area	Dose applied under occlusion ($\mu g/cm^2$)
301	18·2	6850	540	7·9	7·4
302	17·0	6540	493	7·5	8·1
304	22·0	7770	501	6·5	8·0
Mean...	19·1	7050	511	7·3	7·8

* Calculated from 9·9 × body weight $(g)^{2/3}$.

mental conditions throughout the study and to avoid radioactive contamination, the pigs were kept in a state of narcosis with pentobarbitone. The room temperature was 28–30°C and the pigs had a body temperature of 36–37°C.

Samples of serum were taken at intervals of 2, 4, 6, 8 and 10 hr after application of the test material and at the end of the exposure period the pigs were killed. The dressing (including any test solution still present) was removed, together with the skin and subcutis as a whole, in order to eliminate any contamination of the subsequently dissected organs and tissues. The test solution still present under the dressing was extracted quantitatively. The underlying skin was thoroughly washed with non-radioactive detergent solution and water. From this skin (and subcutis), six samples each 20 mm in diameter were punched out to determine the adhering activity and to measure the depth to which [^{14}C]BLANKOPHOR BHC penetrated into the skin. Levels of ^3H and ^{14}C activity were measured in the tissue samples, in the homogenized gastro-intestinal tract and its contents, and in plasma, urine (collected during the exposure period and at the end of the test) and bile, as well as in the water condensed from the freeze-dried tissue samples.

Measurement of radioactivity. Radioactivity was measured by liquid scintillation counting, double or triple determinations being performed on all samples. Prior to assay for ^3H and ^{14}C, the samples were freeze dried. Urine and bile were pipetted on to cellulose. The samples were then combusted in an oxygen atmosphere in an Oxidizer 306 (Packard Instruments, Frankfurt/M.). The separated ^3H and ^{14}C samples were counted in liquid scintillators. Plasma and the water of condensation from the freeze-dried tissue samples were pipetted into Instagel (Packard Instruments). The samples were counted in either a Nuclear Chicago Mark 1 or a Packard Model 3313 liquid scintillation spectrometer, at a temperature of +2°C. Counting efficiencies were determined by external standardization. The data obtained for the double [^3H and ^{14}C]-labelled samples were processed and analysed in a computer specially programmed for such samples.

The ^{14}C activity measured in the organs and tissues is expressed in this paper as unchanged BLANKOPHOR BHC equivalents in ppb (ng/g), calculated by dividing the activity measured in a tissue sample (dpm/g) by the specific activity of the applied labelled compound (dpm/ng). The total amounts of activity in the animal body (expressed as a percentage of the applied ^{14}C activity) were determined by summing the total activity present in each organ and tissue. Tissue weights not accessible by direct weighing were estimated on the basis of values given in the literature (Altman & Dittmer, 1964; International Commission on Radiological Protection, 1974) as follows: muscle c. 40% of body weight, fat c. 20%, skin (less subcutaneous fatty tissue) c. 4%, blood collected terminally 3·2% (equivalent to 50% of total blood), connective tissue 2·3% and remaining tissues c. 20%. The equivalent concentrations in the 'remaining tissues', not determinable for preparative reasons, were based on the concentrations determined in the muscle of the pigs. This procedure seems to be justified in the light of results obtained from range-finding whole-body autoradiographic studies on the rat. These studies revealed that at late points of time (24 hr after application), the concentrations in the 'remaining tissues' were not higher than those in the muscle.

In anticipation that absorption of BLANKOPHOR BHC would be slight and the resulting concentrations in the organs low, precautions were taken to eliminate the risk of contamination with radioactivity by processing two untreated pigs in an alternating series with the three pigs to which the radiolabelled FWA was applied. No contamination whatsoever was noted. All assayed samples from these pigs showed values below the limit of detection which, according to the nature of the sample, sample weight and method of measurement, ranged from 0·1 to 1 ppb. The amounts of ^3H activity present terminally in the animal body, bile and urine, i.e. after a 10-hr period of absorption, are expressed as percentages of applied ^3H activity.

Localization of ^{14}C activity at various levels in skin. After removal of the deeper parts of the subcutis, six segments (c. 3 × 3 mm) were cut out of the skin beneath the site of application and immediately deep-frozen using liquid nitrogen. Next, vertical native frozen sections 10 and 40 μm thick were cut at about −25°C, dried (to remove ^3H activity) and autoradiographed. The 40-μm sections were placed on Agfa-Gaevert Curix MR 4 X-ray film and the 10-μm sections on slides coated with Kodak AR 10 stripping film. The sections were in close contact with the photographic emulsion and were exposed at −20°C for up to 7 wk. After exposure, the section and emulsion were separated, the sections were stained with haemalum and eosin and the X-ray and stripping films were developed. Each stained section was then recombined with its autoradiogram and examined microscopically.

Table 2. [^{14}C]*BLANKOPHOR BHC equivalents in the organs and tissues of pigs after dermal application of 4 mg to approximately 500 cm² of skin under an occlusive dressing for 10 hr*

Organ/tissue	Level (ppb) of ^{14}C as BLANKOPHOR BHC in pig no. 301	302	304
Liver	1·27	1·01	0·70
Kidney	0·59	0·31	0·20
Lung	<0·3	<0·3	<0·3
Skin	<0·3	<0·3	<0·3
Muscle	<0·13	<0·13	<0·13
Connective tissue	<0·5	<0·5	<0.5
Renal fat	<1	<1	<1
Subcutaneous fat	<1	<1	<1
Erythrocytes	ND	<0·2	<0·2
Plasma	<0·12	<0·12	<0·12

ND = Not determined

From the skin at the application site on one of the pigs (no. 302), two cylindrical skin specimens 3 mm in diameter were punched out and mounted on to the cold stage of a rotary microtome (Type 1130; Jung AG, Heidelberg) in a cryostat (Dittes, Heidelberg). A special adjusting device was used for getting sections parallel to the skin surface, and native frozen sections of the stratum corneum and the epidermis were cut at 10 μm. As minimal levels of ^{14}C activity were expected in the deeper layers of the skin, section thickness was afterwards extended to 40 and 80 μm. The cut sections were combusted in an oxygen atmosphere by the Micro-Mat technique (Wegner & Winkelmann, 1973) and the $^{14}CO_2$ was absorbed in phenylethylamine and counted in liquid scintillators.

RESULTS

Absorption of ^{14}C activity within 10 hr

Since the biological samples were assayed directly for radioactivity without prior extraction, the results relate to unchanged BLANKOPHOR BHC and its metabolites. No ^{14}C activity was detected in any of the serum samples taken from the three pigs at intervals of 2, 4, 6, 8 and 10 hr after application. Throughout the exposure period, therefore, the serum levels of BLANKOPHOR BHC or its metabolites were less than $0·12 \times 10^{-6}$ mg/ml (0·12 ppb). The concentrations of ^{14}C activity in the organs and tissues of the three pigs were converted to BLANKOPHOR BHC equivalents and are presented in Table 2. The concentrations in the tissues analysed were below the limit of detection except in the liver and kidney. The highest equivalent concentration found was 1·3 ppb in the liver of one pig. Conversion of these concentrations to the amounts of activity present in the organs and tissues and the determination of ^{14}C activity in the gastro-intestinal tract and its contents and in the urine and bile provided the values for absorbed activity given in Table 3. There was no faecal excretion during the exposure period.

As the limit of detection for [^{14}C]BLANKOPHOR BHC was exceeded in only a few organs, the symbol < is prefixed to the values in Table 3, with the exception of those given for urine. The total amount of activity detected in the animal body (excluding the application site) plus that excreted in the urine during the exposure period and detected in the urine and bile at the end of the exposure ranged from 0·17 to 0·22%. Therefore, dermal absorption is estimated to have amounted to a maximum of approximately 0·2% of the BLANKOPHOR BHC dose applied to the skin.

The application site (skin and subcutis, together with the occlusive dressing and the residual test solution) isolated completely at the end of exposure was assayed for ^{14}C and ^{3}H activity. Of the ^{14}C activity, a good 50% was present in the detergent solution and approximately 25% was present on/in the skin at the application site. Thus more than 75% of the applied ^{14}C activity was recovered experimentally. This assay proved to be difficult, particularly because a quantitative recovery of the applied test solution distributed over such a large area of skin was impossible, because only a few samples could be taken from the skin and because activity that might have been

Table 3. *Absorbed radioactivity detected after dermal application of 4 mg* [^{14}C]*BLANKOPHOR BHC to approximately 500 cm² of skin for 10 hr under occlusion*

Pig no.	Radioactivity (% of applied dose) detected in: Organs and tissues	Gastro-intestinal tract (including contents)	Bile	Urine	Total activity (% of dose)
301	<0·15	<0·006	<0·0002	0·04	<0·20
302	<0·14	<0·005	<0·0002	0·02	<0·17
304	<0·18	<0·007	<0·0002	0·03	<0·22

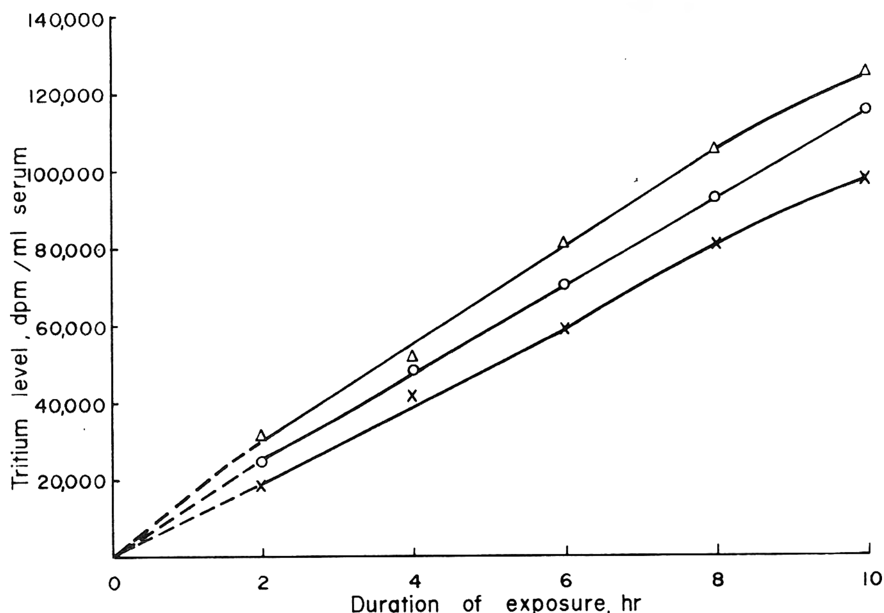

Fig. 1. Tritium levels in the serum of three pigs during dermal exposure for 10 hr to a ^3H_2O-containing detergent solution under an occlusive dressing.

adhering to the polythene film of the occlusive dressing was not determined. A recovery of more than 75% therefore seems most satisfactory. It also proves that throughout the exposure period an adequate amount of tracer was in direct contact with the skin under the occlusive dressing.

Measurements of ^3H activity

Tritiated water was added to the test solution to check whether the experimental design would guarantee dependable dermal absorption of a compound over an exposure period of 10 hr. Furthermore, it was necessary to determine the extent and speed of water absorption in the pigs in order to ensure that the results could be extrapolated to the human situation.

From Fig. 1, in which the activities of tritium determined in the serum of the three pigs are plotted in relation to time, it is evident that absorption of the water through the skin began immediately after application and was largely proportional to time throughout the 10-hr period of contact. Variation between the three animals was comparatively slight.

When the organs and tissues dissected from the pigs at the end of the 10-hr exposure period were freeze-dried, the ^{14}C activity was retained quantitatively in the dried tissues and there was no indication of any volatilization having taken place. On the other hand, the tritium activity was present in the water condensed from the freeze-dried tissues. Its concentration corresponded to the 10-hr value shown in Fig. 1, and thus confirmed the results of the measurements of activity in serum. From this concentration, and assuming the water content of each pig to be 60–65% (w/w), the tritium content of the animal and hence the amount of water absorbed from the application site and retained in the body was calculated to be between 25·3 and 29·0% (mean 27·3%) of that applied. On adding to this figure the minimal amounts of ^3H activity in urine and bile ($\leq 1\%$ in each), an absorption figure of approximately 30% was obtained.

About 50% was found in the residual test solution removed by suction from beneath the occlusive dressing and in the skin washings. Therefore, about 80% of the applied tritium activity was recovered altogether, a value in good agreement with the recovery of ^{14}C activity. As in the latter case, the rest of the tritium activity is considered to have been lost during the removal of the occlusive dressing and the subsequent washing of the skin of the pig. The ^3H activity was not determined in the skin from the site of application; because of the relatively good diffusibility of the water, the content of ^3H-activity at this site was estimated to be negligible.

Localization of ^{14}C activity in the skin after the 10-hr exposure period

The results presented above indicate that at the end of the exposure period about 25% of the topically applied ^{14}C activity was still present on/in the skin. It was not possible to remove this activity by washing and swabbing the epidermis, which was slightly swollen as a result of being covered for 10-hr with an occlusive dressing. Therefore, the distribution of ^{14}C-activity in the skin was analysed. At the end of the exposure period, the mean level over the entire area of skin (c. 500 cm^2) was about 2 μg FWA/cm^2 or 0·15 μCi $^{14}C/cm^2$.

Localization of ^{14}C activity at various levels of the skin

All autoradiograms of sections of skin from the site of application of [^{14}C]BLANKOPHOR BHC showed massive accumulation of activity on and in the stratum corneum, which had become partially squamous as a result of being covered with the occlusive dressing. Under the stratum corneum, in the epidermis, corium and adhering subcutis, only a light silver-grain fogging of the photographic emulsion was apparent. There was no indication that the FWA had penetrated along the bristles down into the hair

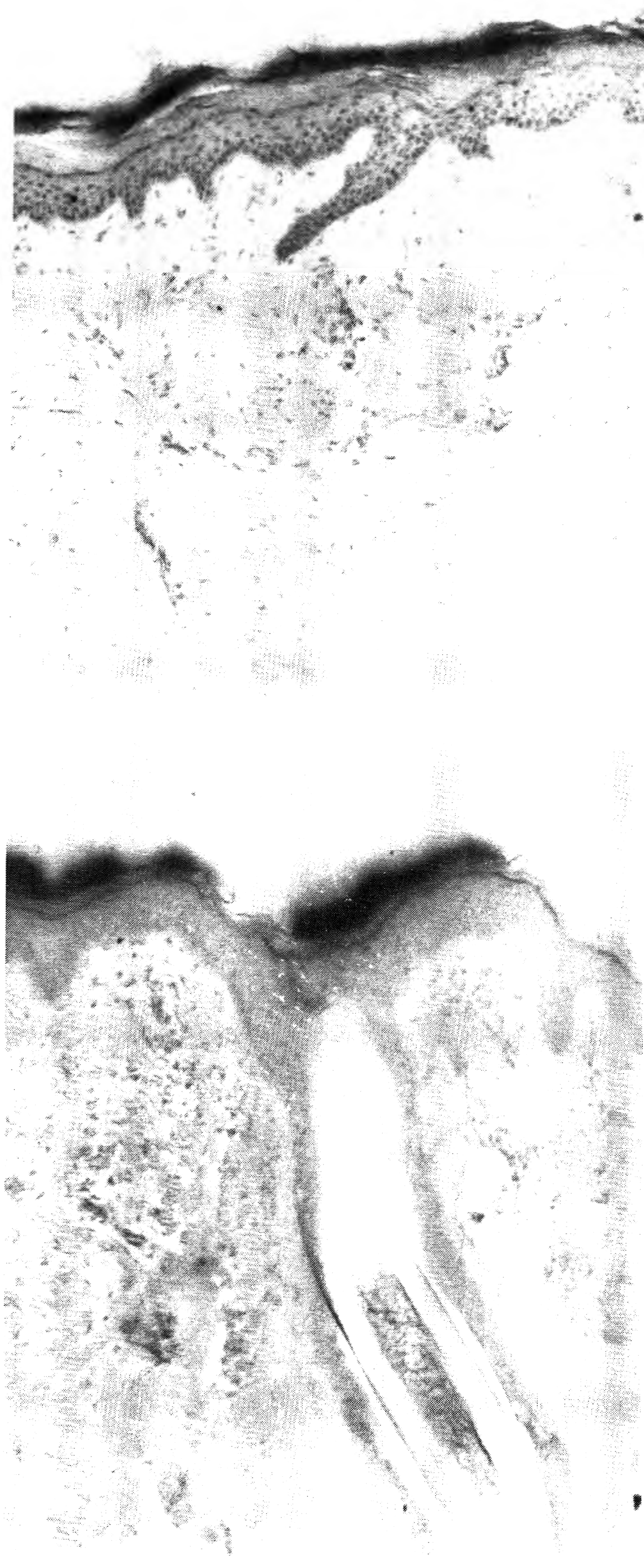

Fig. 2. Autoradiograms and underlying histological sections through the skin of a pig after dermal application of 4 mg [^{14}C]BLANKOPHOR BHC to a skin area of 500 cm^2 under occlusion for 10 hr. The blackening above the stratum corneum indicates substantial accumulation of ^{14}C activity. Frozen section (10 μm) stained with haemalum and eosin. × 148 (above) and × 165 (below).

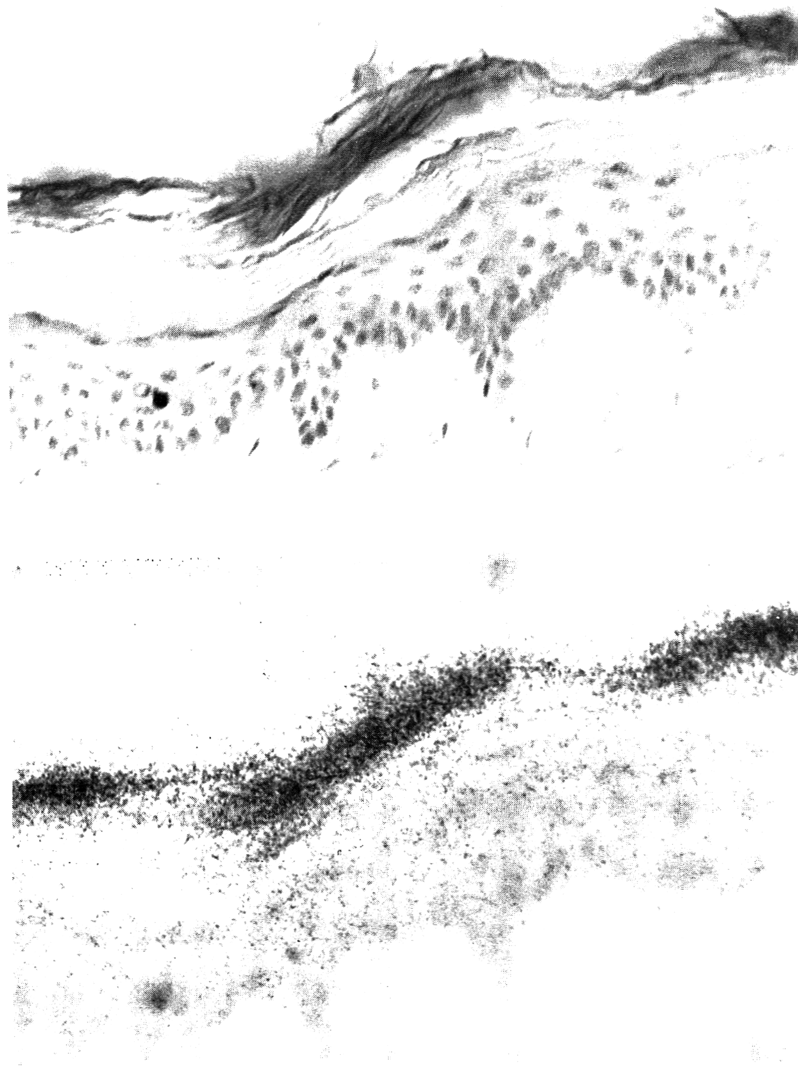

Fig. 3. Histological section and corresponding autoradiogram through the skin of a pig after dermal application of 4 mg [^{14}C]BLANKOPHOR BHC to a skin area of 500 cm^2 under occlusion for 10 hr, showing (above) sharp definition of the histological section and (below) sharp definition of the autoradiogram. The blackening above the stratum corneum indicates substantial accumulation of ^{14}C activity. Frozen section (10 μm) stained with haemalum and eosin. × 400.

follicles. Figures 2 and 3 illustrate some typical findings relating to the distribution of ^{14}C activity in the pig skin.

The results of the autoradiographic studies were supplemented by quantitative determinations of the ^{14}C-activity in different layers of the skin of pig no. 302. With increasing distance from the surface, a rapid decline of the ^{14}C activity in the skin was noted. The levels of activity at a depth of 250 μm were found to be 1000 times lower than those in the stratum corneum (the mean of the first two 10-μm sections). The concentrations in the deeper layers of the corium and in the subcutis were close to or below the limit of ^{14}C detection in these sections. With this method, the steep decline of activity below the stratum corneum was less pronounced than was demonstrated morphologically in the autoradiograms, because of the microfolds and pores of the stratum corneum, which extended to a depth of about 200 μm. However, the decline in activity by a factor of 1000 within the space of about 250 μm confirms the autoradiographic finding that BLANKOPHOR BHC is localized almost exclusively in the stratum corneum even under the extreme conditions of the occlusive dressing.

DISCUSSION

In these studies, the dermal absorption of the FWA was of foremost interest. To confirm the suitability of the experimental conditions, however, dermal absorption of tritiated water was studied simultaneously. The rate of absorption of the water (Fig. 1) led to the absorption in 10 hr of nearly 30% of the applied ^{3}H activity (contained in 20 ml detergent solution). This was equivalent to approximately 7 ml water calculated for a skin area of 500 cm^2, or to 1·4 mg water $cm^{-2}\,hr^{-1}$. In man, we recorded in earlier studies (involving exposure of a skin area of 275 cm^2 for 4 hr), a water absorption of 1·45 mg $cm^{-2}\,hr^{-1}$ (L. A. Wegner and K. Patschke, unpublished data 1961), a value in good agreement with that obtained in similar studies conducted by other authors (Pinson & Langham, 1957). We therefore conclude that the experimental conditions used were suitable for dermal absorption studies, and that it is permissible for the results obtained in pigs to be extrapolated to man.

Those results showed that a maximum of about 0·2% of the dermally applied dose of BLANKOPHOR BHC was absorbed within 10 hr. This corresponds to a maximum absorption of 8 $\mu g/500\,cm^2$ in 10 hr or 1·6 ng $cm^{-2}\,hr^{-1}$, compared with a figure of 1·45 mg $cm^{-2}\,hr^{-1}$ for the absorption of water under the same conditions.

Because of this very slight absorption of the FWA, the levels of ^{14}C activity in the organism, expressed as BLANKOPHOR BHC, in no tissue exceeded 1·3 ppb, a level reached only in the liver of one animal. In most of the organs analysed, the values were below the limit of detection of the highly sensitive method used for the radioactivity determinations. In plasma, the concentrations were less than 0·12 ppb.

Studies of the localization of residual ^{14}C activity on/in the skin at the end of the experimental period clearly showed that the FWA has a marked affinity for the stratum corneum (Figs 2 & 3). The stratum corneum is dyed by FWAs like a textile fabric; hence the persistent fluorescence of the skin observed after use of FWA-containing detergents. The stratum corneum, no more than 50 μm thick, definitely acts as an effective barrier to the penetration of BLANKOPHOR BHC into the deeper skin layers and into the organs and tissues.

The ^{14}C concentrations in the basal cells of the epidermis and the cutis were close to or below the limit of detection for skin sections. This finding confirms the already mentioned minimal absorption of ^{14}C activity. Starting from the basal cylindrical cells of the epidermis, the skin undergoes constant regeneration, and it is reasonable to conclude, therefore, that the activity still present in the skin at the end of the 10-hr experimental period will not be absorbed or will be absorbed only to a negligible extent (compared with the amount recovered in the tissues after 10 hr), and that it will be desquamated together with the scales of the stratum corneum in the process of skin regeneration. Similar findings with other FWAs have been reported (Gloxhuber, 1972; Jadassohn & Schaaf, 1970; Luckhaus & Löser, 1975).

Therefore, delayed absorption of the BLANKOPHOR BHC retained on the stratum corneum is unlikely and there should not be any change in the value given above for the total absorption of the FWA, namely a maximum of approximately 1·6 ng $cm^{-2}\,hr^{-1}$. As the skin surface becomes squamous after being covered for 10 hr with an occlusive dressing, and the accompanying hydration of the epithelium creates optimal absorption conditions (Idson, 1977), this value (1·6 ng $cm^{-2}\,hr^{-1}$) is considered to represent the upper limit of dermal absorption for BLANKOPHOR BHC. Furthermore, this result was obtained with a BLANKOPHOR BHC content of 0·02% in the detergent solution; in the customary detergent solutions, the FWA concentration is only 0·001%.

Usually, during the washing process, only the hands become wetted with the suds. The ratio of the skin area of both hands (volar and dorsal sides extending to the wrists) to the total skin area of standard man is about 0·05. When, for the purpose of comparison with the results of other authors, the dermal absorption rate of $<1{\cdot}6$ ng $cm^{-2}\,hr^{-1}$ calculated for BLANKOPHOR BHC is related to 5% of the total skin area of a person weighing 70 kg, it can be estimated that a maximum of 1·4 μg of the FWA will be absorbed hourly through the skin of both hands moved in suds containing BLANKOPHOR BHC.

From this exceptionally low level of dermal absorption, it is concluded that the use of this FWA in detergents poses no hazards. With the extremely low concentrations in the tissues there is no likelihood of any systemic side-effects.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

The Surveillance of Food Contamination in the United Kingdom. The First Report of the Steering Group on Food Surveillance. Ministry of Agriculture, Fisheries and Food. Food Surveillance Paper No. 1. HMSO, London, 1978. pp. v + 38. £0.90.

This Steering Group was set up in 1971 to keep specific food-contamination problems under review, and to arrange for any analytical surveys that it may consider necessary. Much of its first report is devoted to the problems of heavy metals, among which lead, cadmium and mercury have received the most attention. Results obtained since the reports of the Working Party on the Monitoring of Foodstuffs for Heavy Metals (*Cited in F.C.T.* 1974, **12**, 139; *ibid* 1976, **14**, 491) have given no particular grounds for concern, but have indicated the desirability of certain more detailed studies.

As children under 1 year old may be most sensitive to the toxic effects of lead, an investigation has been started into the diets of infants in areas where lead piping predominates and the water is more plumbosolvent than normal. Almost half of the cadmium present in the diet is contributed by cereals and vegetables, and cadmium uptake from the soil is now being studied. Crops grown on land treated with sewage sludge may have a particularly high cadmium content, and a survey is therefore being undertaken of the diet of people who consume food produced on such land. The need for and extent of use of organomercurial dips for controlling aspergillosis in egg hatcheries was also deemed to require investigation.

A preliminary survey of arsenic in the total diet indicated a mean weekly intake of not more than 0·8 mg, of which about half is derived from fish and shellfish. However, this is mostly in the form of organically-bound arsenic, which does not accumulate in the tissues. For copper, selenium, nickel and zinc, mean daily intakes were estimated (on the basis of 1974 total diet samples) to be 1·7, 0·06, 0·02 and 11·0 mg, respectively. Analyses of specific foods for these metals and for tin, iron and manganese have also been conducted, and results are presented in the report.

The nitrate and nitrite content of foods was another topic reviewed by the Steering Group. More information was considered necessary on the levels of each in baby foods, the effects of storage, processing and cooking on the conversion of nitrates and nitrites, and the amounts present in the total diet. Preliminary analyses have confirmed published reports that the highest nitrate levels occur in certain vegetables, such as spinach and celery. A survey of polychlorinated biphenyls in fish and shellfish indicated that levels in freshwater species were considerably below those reported from some other countries, and the problem should diminish in view of the controls now imposed on these compounds.

Taint produced in poultry meat by chloranisoles was concluded to be a problem of food quality rather than of consumer health, as only three of the samples analysed contained detectable levels, all in the region of 0·002 mg/kg. Of the many different residues that may occur in organic wastes used as animal feeds, the antibiotics, hormones and arsenical compounds were considered unlikely to present a human health hazard. Further monitoring was recommended, however, to confirm the absence of significant residues of coccidiostats and chlorophenols in human food, and a study has been arranged of the effects on food when dinitolmide, sulphaquinoxaline, robenidine and nifursol (all widely used in poultry husbandry) are ingested by cattle.

Comprehensive surveys of mycotoxins and antibiotic residues in foods are now being organized by the Steering Group. Consideration is also being given to the monitoring of pesticide residues in foodstuffs at the retail level. In view of EEC pressure for the restriction of residues of veterinary preparations and additives, such as growth promoters and metabolic stimulants, used in compound animal feeds, the requirements for monitoring such residues are being explored. A Special Survey Unit, formed within the Food Science Division of MAFF, will be responsible for organizing and executing special surveys of residues in foods.

Survey of Vinyl Chloride Content of Polyvinyl Chloride for Food Contact and of Foods. The Second Report of the Steering Group on Food Surveillance. The Working Party on Vinyl Chloride. Ministry of Agriculture, Fisheries and Food. Food Surveillance Paper No. 2. HMSO, London, 1978. pp. iv + 16. £0.60.

The Working Party on Vinyl Chloride was set up in 1973, following an FDA proposal to ban the use of PVC resins for packaging alcoholic beverages (*Food Chemical News* 1973, **15**(9), 42). Its objective was to provide information on food-contact uses of PVC, vinyl chloride (VC) levels in PVC and in food, factors affecting the latter, and the maximum likely intake of VC from food.

The three main types of PVC for food packaging were identified as bottles, rigid film (foil) and flexible film, of which only the last category had a VC content consistently below 1 ppm throughout the study. Of PVC bottles, only 20% contained below 10 ppm VC in April 1974, but the proportion increased steadily, until by February 1977 all were below 1 ppm. A similar but less marked trend was observed with rigid

film, where the percentage of samples having a VC content below 5 ppm increased from 57 to 95% over the 3 years These reductions were attributed chiefly to changes in manufacturing temperature and improved blending techniques. Levels in foods were dependent on storage time and temperature, and on VC levels in the polymer. Significant items of the diet that are generally packaged in PVC were found to be concentrated fruit drinks, cooking oil and soft margarine. and by 1977 VC levels had been reduced to below 0·002 ppm in all these foods, except for two of 13 fruit drink samples which contained up to 0·01 ppm. On the basis of these findings the maximum daily VC intake per person was estimated to be 1·3 μg in early 1974, and to have fallen to 0·1 μg by May 1976.

These findings were considered by the Carcinogenesis Sub-Committee in April 1977. It was concluded that co-operation between government and industry had proved an effective means of controlling and reducing VC levels, and industry was encouraged to continue its efforts in this direction. The Sub-Committee's comments were endorsed by the Toxicity Sub-Committee, which drew attention to the exemplary approach of the Working Party. The FACC also expressed its appreciation and satisfaction with the progress made by the Working Party in reducing VC levels in PVC and in food.

Criteria (Dose/Effect Relationships) for Cadmium. Report of a Working Group of Experts prepared for the Commission of the European Communities, Directorate-General for Social Affairs, Health and Safety Directorate. Rapporteur R. Lauwerys. Pergamon Press, Oxford, 1978. pp. vii + 202. £9.50.

The Commission of the European Communities decided to initiate a work programme to assess the effects on health of a number of common pollutants. The first fruits of this programme have now appeared in the form of a report containing the opinions of a group of invited experts on the toxicological status of cadmium.

As well as metabolic and toxicological data the report contains information on the levels of cadmium in the air, food and water. Chapters are included on the metal's chemical and physical properties and uses, its occurrence and production and the sources of environmental cadmium pollution. On the basis of these data, the Working Group has tentatively proposed a no-effect level for long-term exposure to cadmium and has briefly outlined the needs for further research.

Any competent review of such a mammoth subject would be welcomed, but a comprehensive and critical review such as the present work, is doubly so. The Working Group met in January and October 1976. Although the bulk of the 500 or more references listed were obviously published before 1977, a small number of 1977 citations in the final report is evidence of further updating. On the whole, the review is well written and general presentation is excellent throughout. The 12-page summary at the beginning of the report facilitates the identification of the important results and helps to compensate for the absence of an index.

Although the data available on most materials are still scanty in the extreme, there are certain topics on which a wealth of information has already accumulated. As the present work testifies, it is exceedingly difficult to keep up-to-date with the toxicological status of these 'popular' materials. For this reason further publications in the present series will be eagerly awaited.

Environmental Health Criteria 1: Mercury. WHO Task Group. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1976. pp. 131. Sw.fr. 14.00 (available in UK through HMSO).

This small volume was the first of the series of books produced as part of the Environmental Health Criteria programme of WHO. The primary purpose of the programme is to compile and assess the data available on the hazards to man's health associated with exposure to environmental pollutants, especially persistent chemicals, and hence to provide scientific guidelines for setting exposure limits for such chemicals. On this basis, one might be forgiven for assuming that the present volume is merely an updating of the excellent book by L. Friberg and J. Vostal entitled *Mercury in the Environment* (*Cited in F.C.T.* 1973, **11**, 661), but this is not so. Whereas the latter is a comprehensive and exhaustive treatise on the subject of mercury toxicity, the former has no such aspirations and concentrates only on data directly relevant to the assessment of human exposure and to the establishment of dose-effect relationships. Thus, much work on experimental animals has been omitted and the environmental transformation and transport of mercury are treated fairly briefly. Even so, space restriction has forced the authors to pare down much of the information to the barest essentials and this approach has resulted in a commendably concise presentation which does not deviate from the stated aims of the WHO programme. There are particularly succinct chapters on the absorption and metabolism of mercury and on clinical and epidemiological studies in man, and in the last chapter the health risks to man from exposure to elemental mercury and its inorganic and organic compounds, particularly the alkyl derivatives, are evaluated in the light of those clinical and metabolic studies.

The text is supported by a fairly extensive list of references to provide a most useful little book which enables its reader to cover relatively quickly the greater part of the significant data on mercury toxicity.

Principles and Procedures for Evaluating the Toxicity of Household Substances. NAS Publication 1138, National Academy of Sciences, Washington, DC, 1977. pp. vi + 130. $7.00.

This publication first appeared in 1964, when it dealt primarily with acute toxicity and was intended to aid the FDA in fulfilling its responsibilities under the Federal Hazardous Substances Act. Because of

substantial methodological advances in toxicology, and the extension of concern to chronic toxic effects, the Consumer Product Safety Commission (now responsible for the Act) made a request in 1976 for the preparation of a new edition. The principles and procedures this describes are applicable to the testing of a wide range of chemicals besides those for household use, and should be of help to toxicologists in a variety of roles.

Separate chapters discuss toxicity tests by ingestion (acute, subchronic and by aspiration), dermal and eye application (including acute toxicity, percutaneous penetration, irritation, phototoxicity, pigmentation changes, contact sensitization and urticaria) and inhalation, as well as studies on chronic toxicity and carcinogenicity, mutagenicity, reproductive and teratogenic effects, and behavioural toxicity. Appendices specify the regulations under the above Act, discuss animal husbandry, and suggest appropriate haematological and clinical-chemistry studies and organs that should undergo pathological examination.

The report adopts the prevailing view that when exposure of pregnant women is possible, chronic rodent studies should start *in utero* and continue until survival of the F_1 generation is down to 20%. It suggests that the upper dose level in such studies should produce some signs of toxicity but should not greatly alter normal physiological function. Prior pharmacological studies will indicate the extent to which a substance may accumulate in different organs and produce unexpected toxicity, or may cause metabolic overloading at high dose levels. On the subject of mutagenicity, it is considered that negative results in at least three of five tests for chromosomal mutation and two of four tests for point mutation give "moderate confidence" of non-mutagenicity in man. Behavioural toxicity is recognized as a young science, but studies of effects on the circadian cycle of activity and operant behaviour are recommended for initial work in this field.

Prostaglandin Research. Organic Chemistry: A Series of Monographs. Vol. 36. Edited by P. Crabbé. Academic Press Inc. (London) Ltd., 1977. pp. vii + 341. £18.50.

"Two things fill my mind with increasing wonder and awe..."

Thus Kant on the "starry heavens" and morality. Had he lived as we do at the dawn of biological understanding, his mind must surely have been unbalanced at the awesome prospect. How will we ever understand the seemingly infinite intricacies of biochemical mechanisms? This book adds to the sense of wonder and at least indicates that the prospect of understanding is feasible.

It is over forty years since the first hint of pharmacologically active 'messengers' in prostatic secretion was obtained and over twenty since the initial successes in chemical identification. Since then these bizarre compounds have become what is arguably the fastest growth area in pharmaceutical research and have given rise to a revolution in our perception of cellular influence and control. Happily there are minds equal to the task, and this masterly volume includes contributions by some of them.

It is a book for anyone who wants to know the basic facts (as far as we know them) about prostaglandins. It deals logically and lucidly with their history, their identity, their occurrence, their function and their chemistry. It is written with a clarity indispensable to a proper display of such a complex topic—and will certainly serve to recruit new minds to the cause.

No toxicologist can afford to ignore the prospect that prostaglandins play a crucial role in toxicity and, although it is probable that most toxicologists are, as yet, ill-equipped to make major contributions to the field, this book could be their salvation. It is strongly recommended.

Developments in Food Analysis Techniques—1. Edited by R. D. King. Applied Science Publishers Ltd., London, 1978. pp. x + 323. £25.00.

Although the methods covered in this book are mainly well established and widely used, it does provide a valuable review of developments up to 1976 (including some references dated 1977). One damper on radical changes in the field of food analysis is the legal need for the results to correlate with those of established methods. Any technical innovation must have clear advantages over existing methods and it must be accepted by food analysts. Inevitably, some of the long-established methods such as the Kjeldahl for nitrogen and the Lane and Eynon method for reducing sugars remain the most widely used.

The chapters have all been contributed by experts in food analysis currently working in British laboratories. Chapters 1, 2 and 9 deal, respectively, with the methods available for determining vitamins, nitrogen and protein, and carbohydrates. All three are useful surveys of extraction, isolation and analysis procedures but they give little indication of possible future trends. Although there are many references to high-pressure liquid chromatography and gas chromatography throughout the book, chapters 4 and 5 are devoted exclusively to these techniques. As well as outlining methods, they provide information on the preparation of columns and derivatives, illustrations of some typical separations and a useful list of references. Chapter 7 deals with ion-selective electrodes, summarizing the most important methods and describing the techniques used for specific ions. The use of enzymes in food analysis is reviewed in chapter 6, which gives a number of interesting examples of the applications of this method.

Chapter 10, on atomic absorption lists the apparatus, methods and possible applications of this spectroscopic technique with few details of its practical application. The costs, the advantages and the limitations of automating analytical processes are thoroughly expounded in chapter 8. Full details of automatic analysis at the Laboratory of the Government Chemist are given and clearly illustrated by schematic diagrams. An extensive mathematical treatment of the various problems associated with the role of water in foodstuffs is provided in chapter 3, which will prob-

ably have rather limited appeal; certainly it is of little practical value to toxicologists.

The book offers a very useful and readable coverage of current practices to those without a great deal of experience in food analysis, but will be less valuable to the experienced food analyst. Subsequent volumes could be improved by an expansion of the coverage of high-pressure liquid chromatography and ion-selective electrodes.

A Colour Atlas of Histological Staining Techniques. By A. Smith and J. Bruton. Wolfe Medical Publications Ltd., London, 1977. pp. 192. £5.00.

The authors of this book, another in the 'colour atlas' series, approach their subject from two different angles, illustrating in the first half of the volume the results that can be obtained by the use of the staining techniques that they describe in the second half. Thus, the first part consists of over 100 well-presented coloured photomicrographs of the more popular techniques for mucins, glycogen, lipids, nervous tissue and pigments. Opposite each colour plate is a brief legend, which names the stain, indicates the tissue elements demonstrated and refers to the page in part 2 on which the description of the working technique can be found. In this latter section, the instructions for each staining or impregnation technique outline, in a simple step-by-step way, the solutions required, the procedure to be followed and the results to be expected. Finally a brief part on tissue processing and sectioning is followed by a list of general references and an adequate index.

This does not pretend to be a textbook. Its prime purpose is to help the student who has limited facilities for histology, and little guidance, to produce a stained product, which he can then compare with the coloured photomicrograph. What it will not tell him is what to do when the product does not match the picture!

Rat Hepatic Neoplasia. Edited by P. M. Newberne and W. H. Butler. The MIT Press, Cambridge, MA, 1978. pp. xii + 288. $22.50.

This book resulted from a Workshop on Rat Liver Neoplasia held at Woods Hole, MA, in October 1974. After a lapse of 4 years, is such an account worth reading? Emphatically yes (despite an above-average quota of typographical errors). It would be a mistake to imagine that fresh solutions to the problems of rat-liver neoplasia have arisen in the interim. Although advances have occurred in certain areas since the time of the Workshop, this book still presents an excellent and remarkably up-to-date description of the various facets of the subject.

The introduction is followed by a clear account of the embryology of the rat liver and of the effects of ageing on this organ. Next come the essentials of the characterization of hepatic nodules, probably the most complex and controversial area in the whole field of hepatic neoplasia. The excellent and incisive analysis presented here gives a useful overview; it is perhaps unfortunate that the author did not update the material at the time of publication, but this would admittedly have involved substantial effort in such a rapidly advancing field.

The chapter on microsomal-enzyme systems and drug toxicities is sadly dated. Nevertheless, it points out the fundamentals of recent advances in the thinking on this subject. The morphological aspects are covered very effectively, perhaps more clearly than in any comparable publication. One chapter deals with sequential cellular alterations during hepatocarcinogenesis and two others review thoroughly the morphological features of rat hepatic neoplasia, demonstrated both by light and by electron microscopy. There is some discussion of hepatocellular growth *in vitro*, its control and characteristics. A section on the immunology of rat hepatic neoplasia provides a useful basis for anyone interested in this aspect of the problem. Finally, the discussion of the effects of diet on chemical carcinogenesis in the rat-liver model indicates the potential problems facing those who take the diet of their experimental animals for granted.

Each chapter is followed by a discussion section. One is faced with topics that have been the subject of debate for many years and, more recently, have caused confrontations at regulatory hearings and in lawsuits concerning alleged carcinogenic pesticides and other products. The central issue is the interpretation of 'lumps', 'nodules', 'hyperplastic lumps' and 'hyperplastic nodules', as against actual neoplasms. What emerges very clearly is the fact that the decision at a Workshop sponsored by the National Cancer Institute to replace the term 'hyperplastic nodule' by 'neoplastic nodule' was not only premature, but also misleading. It may have proved convenient for lawyers and legislators but was not justified by the scientific evidence. Another question discussed in this book is whether the rat or the mouse is the more suitable for screening for carcinogenic potential. The need to distinguish the objectives of research in experimental carcinogenesis from those in screening new products for possible carcinogenicity is highlighted. The prevailing view seems to be that the rat is somewhat superior to the mouse simply because we have a better understanding of the critical features, and perhaps the diagnostic criteria, in the rat than in the mouse. What emerges is the need for more skilfully planned and directed work, particularly to clarify the mouse model. It is sad that we continue to use these models for screening purposes without having had the opportunity to clarify them.

Perusal of this volume is strongly recommended to anyone wishing to become familiar with the background to this difficult subject.

Nonparametric Statistics. A Contemporary Approach. By R. P. Runyon. Addison-Wesley Publishing Co., Reading, MA, 1977. pp. vi + 218. £6.40.

Those of us who can never quite remember the right place to use a one-tailed test in place of a two-tailed test, or the precise meaning of Type I and Type II errors and the power of a test, would find this an excellent book to have on hand for a quick reminder. These are concepts fundamental to signifi-

cance testing, and to the initial design of any experiment, but without regular use their definition soon becomes blurred. Chapter 1, an introduction to probability, and Chapter 2, on power and power efficiency, cover these points particularly well, in the context of a broad introduction. A work-text format is used, which gives step by step examples for all procedures, and alongside, exercises for immediate reinforcement. At the end of the book, there are worked solutions to these exercises, where answers can be checked and mistakes quickly located.

Following the two introductory chapters, the book is designed in three sections, each based on a different scale of measurement and sub-divided to cover the one-sample case, independent samples, and related samples. The first section describes the nominal scale, a scale based on qualitative variables that differ in kind rather than by 'how much', e.g. dead/alive, male/female, and similar classifications with more than two categories. Such data may also be referred to as frequency data or categorical data. Proportions and percentages are the descriptive statistics most often used to summarize nominal data. The one-sample case would apply to an observed incidence in a test group in relation to a known incidence in the untreated general population accumulated over a period of time. In contrast, the same test-group incidence may be compared with incidence in an untreated control group in the same experiment, when the two groups are regarded as independent samples, and the chi-square test or Fisher's exact test are appropriate. Related samples may concern changes when the same individuals are assessed before and after some treatment. Tests based on signs, those of McNemar and Cochran, are described for these cases.

Ordinal scales, like nominal scales, are based on qualitative variables, but in this case variables to which some order, direction or magnitude has been given. They are typically expressed in terms of rank. In the one-sample case, the Kolmogorov–Smirnov test examines cumulative frequencies to compare the observed distribution with another, theoretical, one; a runs test can be used to see whether a sample was drawn at random from a population. For independent samples, the median test, the Mann–Whitney test and the Wald–Wolfowitz runs test are described for use with two samples, with an extension of the median test and Wilcoxon's one-way analysis by ranks for several samples. For related samples, a sign test and Wilcoxon's signed-rank test are appropriate for paired comparisons, while Friedman's two-way analysis of variance serves in the multi-sample case.

In the third section, interval and ratio scales are discussed. Most commonly, parametric tests of significance are used with these scales. Such tests assume that the parent populations were normally distributed, and there are sometimes doubts about this, especially in the case of derived values (ratios). Alternative nonparametric tests are described. Most of them look rather tedious, and involve enumerating all outcomes as deviant as, or more deviant than, the obtained outcome. In practice, the temptation would be to rank the observations and apply something from the previous section.

Examples are drawn from sociology, an area that is perhaps less bedevilled with tied ranks than biology. More guidance on tied ranks would have been useful. The tables are well planned, showing where possible both one- and two-tailed critical values. Among those prepared specifically for this book are tables A and B, the latter giving the one-tailed critical values of the binomial for various values for P and Q with N from 2 to 49. Another, table G, with nothing in it that cannot be found in any book of tables, tabulates the area under the curve and the height of the ordinate at z so that it holds together and makes sense in a way that more condensed tables often fail to do.

This book could be useful for anyone who needs an introduction to the use of nonparametric statistics, and for the occasional user who has time to forget between applications. Perhaps the most daunting thing about it is its avowed aim "to emphasize...... the possibility of achieving conceptual and computational mastery of the subject matter without a deadening array of complicated formulas and esoteric mathematical proofs".

Writing a Scientific Paper. By V. Booth. The Biochemical Society, London, 1977. pp. 32. £1.00.

This booklet by Vernon Booth is a gem. It has been around for several years but the fourth edition has now appeared. Revisions have been made in the text, and a useful glossary of printers' terms has been added. The essay is clear, concise and lively, and tells you how to write that way.

Every aspect of writing a scientific paper is covered, including where to start, literary style, spelling, punctuation and accuracy. It is packed with useful advice. One example: "Ask yourself often 'Would a reader whose first language is not English understand what I write?'". Parts of the book are written in the imperative for simplicity, but the preface states that this is not intended to be categorical. Examples are used skilfully and deal with many of the common pitfalls of grammar and comprehension. The section on further reading lists a broad selection of aids to better writing.

This essay is fun to read and it is highly recommended to all scientists. If you don't have time to read it all, although that would not take long, do read the parts suggested in the preface. The booklet costs only £1, and discounts are offered on orders of more than ten copies. Orders together with the remittance, should be sent to: The Biochemical Society Book Depot, P.O. Box 32, Colchester CO2 8HP, Essex.

BOOKS RECEIVED FOR REVIEW

Neurotoxicology. Vol. 1. Edited by L. Roizin, H. Shiraki and N. Grčević. Raven Press, New York, 1977. pp. xxviii + 658. $66.00.

Scientists Must Write. A Guide to Better Writing for Scientists, Engineers and Students. By R. Barrass. Chapman & Hall, London, 1978. pp. xiv + 176. £2.95.

Guide to Hygiene and Sanitation in Aviation. By J. Bailey. WHO, Geneva, 1977. pp. 170. Sw.fr. 28.

Stérilisation et Désinfection par les Gaz. By M. Chaigneau. Maisonneuve, Moulins les Metz, 1977. pp. 344. F.fr. 300.00.

The Biogeochemistry of Lead in the Environment. Part A. Ecological Cycles. Edited by J. O. Nriagu. Elsevier/North-Holland Biomedical Press, Amsterdam, 1978. pp. x + 422. Dfl. 155.00.

Information Section

ARTICLES OF GENERAL INTEREST

TRACKING GLUTAMATE LESIONS IN THE NEONATAL BRAIN

Brain damage is the most frequently reported toxic effect of monosodium glutamate (MSG) in neonatal animals (*Cited in F.C.T.* 1977, **15**, 347). The following papers present attempts to define the damage more fully and locate the susceptible areas of the hypothalamus.

Olney *et al.* (*Brain Res.* 1977, **120**, 151) examined the vulnerability of the area postrema, a circumventricular area of the hypothalamus, to damage induced by glutamate. Ten-day-old and adult HA-ICR mice were given a single sc injection of monosodium glutamate (MSG), the dose given being 1, 2, 3 or 4 g/kg for infants and 2, 4 or 6 g/kg for adults. The animals were anaesthetized 3–5 hours later and brain slices were prepared. All animals given MSG showed a lesion of the area postrema essentially identical with that seen in the arcuate nucleus of mice given MSG. The characteristic findings were massive oedema and organelle degeneration of dendritic processes and neuronal somata, and rapid pyknotic changes in the nuclei of affected neurons. The reaction in adults was less severe than that in infants and involved a smaller number of neurons of the area postrema. Since this area adjoins the chemoreceptor trigger zone, stimulation of which gives rise to emesis, it is possible that small MSG doses may stimulate the neurons enough to induce vomiting while neuronal degeneration follows larger doses. This view is supported by evidence from earlier studies showing that a sharp rise in plasma glutamate rapidly induces vomiting in dogs (Unna & Howe, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1945, **4**, 138), monkeys (Olney *et al. J. Neuropath. exp. Neurol.* 1972, **31**, 464) and man (Levey *et al. J. Lab. clin. Med.* 1949, **34**, 1238).

Reynolds *et al.* (*' Toxicol. envir. Hlth* 1976, **2**, 471) induced hypothalamic lesions in neonatal mice by oral doses of 0·5–4·0 mg MSG/kg or 1–2 g aspartame/kg but found that lower doses did not have this effect. The hypothalamic lesions induced by MSG were much more severe than those induced by equal doses of aspartame. However, infant macaques dosed by stomach tube with up to 4 g MSG/kg or 2 g aspartame/kg did not develop any brain lesions detectable at either the microscopic or ultrastructural level. Thus, the susceptibility of the neonatal mouse brain to MSG is not shared by the primate brain. The possibility that the resistance of the neonatal primate brain to MSG reflects the efficiency of either liver metabolism or the blood–brain barrier in these animals remains an open question.

Holzwarth-McBride *et al.* (*Anat. Rec.* 1976, **186**, 185) also treated newborn mice with MSG by the sc route, giving doses of 2·5 g/kg daily on days 5–10 after birth. Examination of the arcuate nucleus of the hypothalamus of these animals showed an 80% decrease in perikarya, and secondary effects of this lesion included endocrine deficiencies, a reduction in reproductive capacity, and obesity associated with small stature. The weights of the anterior pituitary, ovaries and testes were significantly reduced in MSG-treated animals, but adrenal weight was unaffected. No significant changes were seen in the concentration of nerve terminals or dense core vesicles in the contact zone of the median eminence of treated animals. This, taken in conjunction with the extensive destruction of perikarya observed in the arcuate nucleus, suggests that afferent nerves from the arcuate nucleus form only a small portion of the total nerve population of the median eminence. The arcuate nucleus is known, however, to contain a small proportion of dopamine-producing neurons which project into the external zone of the median eminence, and in a further study by the same group (*idem, ibid* 1976, **186**, 197) the effect of the MSG-induced lesion of the arcuate nucleus on catecholamines in that area and in the median eminence of the mouse hypothalamus was followed by means of a histofluorescence technique. A green fluorescence produced by this method demonstrated the presence of the primary catecholamines, dopamine and norepinephrine. The number of fluorescent perikarya in the arcuate nucleus was reduced by about 60% in newborns treated on six successive days with an sc injection of 2·5 g MSG/kg, and fluorescence in surviving neurons was markedly reduced. The intensity of fluorescence in the median eminence was also reduced. When the mice were pretreated with 400 mg pargyline/kg to inhibit monoamine oxidase, fluorescence in both sites was greatly increased both in MSG-treated animals and in controls, although the number of fluorescing perikarya in the arcuate nucleus remained much smaller in the former group than in the latter. Thus, MSG administered to the neonatal mouse appears to destroy a large number of dopaminergic perikarya in the arcuate nucleus.

Contradicting this evidence of MSG neurotoxicity is the report by Heywood *et al.* (*Toxicology Lett.* 1977, **1**, 151) that when food containing 10% (w/w) MSG or drinking-water containing 5% (w/v), providing very high mean daily intakes of 45·5 or 20·9 g MSG/kg, respectively, was consumed by weanling mice for 4 days (from 20 days of age) no lesions were induced in the hypothalamus. Determinations of plasma-glutamate concentrations immediately before the animals were killed showed that there was, nevertheless, a two-fold increase when MSG was added to the food, and a 1·5-fold increase when it was added to the drinking-water. It is concluded that a threshold

level for the neurotoxicity of MSG in the diet has yet to be established in neonates and in mature animals, in spite of the demonstrated effects of MSG administered by sc injection or by gavage.

[P. Cooper—BIBRA]

CADMIUM AND THE KIDNEY

The mammalian kidney is one of the main targets of cadmium (Cd) toxicity. Nephrotoxic effects of Cd in animals and man were described in an earlier review (Grasso, *Fd Cosmet. Toxicol.* 1975, **13**, 470). Much more of the recent work in this field comes from Japan where Cd pollution of water used in rice fields has been implicated as the cause of the painful bone disease, 'itai-itai' (*Cited in F.C.T.* 1977, **15**, 478). Several of the papers cited below discuss the possible involvement of other food constituents in Cd toxicity. In the industrial field, there are many sources of Cd dust (e.g. in battery manufacture), and studies that indicate the early warning signs of Cd intoxication are especially valuable.

Human studies

The effects of Cd exposure on solderers have been reported by Welinder *et al.* (*Br. J. ind. Med.* 1977, **34**, 221). In 21 solderers exposed in a typewriter factory for periods of 1 month to 18 years to a mean concentration of 30 nmol Cd/m^3 in the form of particles of less than 1 μm diameter, Cd concentrations ranged from less than 10 to 440 nmol/litre in blood and from less than 0·5 to 27 μmol/mol creatinine in urine. Individual blood Cd concentrations varied widely with time, whereas individual urinary levels showed less variation. Increasing the time of exposure to Cd significantly increased urinary excretion of Cd. Four other solderers who had received intermittent exposure for 8–20 years had blood levels of 45–150 nmol Cd/litre and urinary levels of 2–20 μmol Cd//mol creatinine, but showed no correlation between blood and urine levels. Most subjects when removed from Cd exposure showed a considerable fall in blood Cd; in 11 subjects the half-life of the element ranged from 25 to 146 days but was usually below 66 days, and blood Cd concentrations eventually reached a plateau. Urinary concentrations of β_2-microglobulin in these workers rose significantly with increasing urinary Cd concentration, when possible age-effects had been eliminated, and might be taken as an early sign of possible renal tubular damage. Kjellström *et al.* (*Envir. Res.* 1977, **13**, 303) studied 240 workers in a Swedish battery factory who were exposed to mean levels of 53 μg Cd/m^3 mainly as oxide dust, and compared their urinary β_2-microglobulin excretion with that of a control group. The mean control excretion of β_2-microglobulinuria was 84 μg/litre and the upper 95% tolerance limit of 290 μg/litre was chosen to give an operational definition of proteinuria. The proportion of workers with β_2-microglobulin levels above 290 μg/litre was 3·4% in the control group and increased with increasing exposure time to 19% of workers who had been employed for 6–12 years and 83% of workers who had been employed for more than 34 years. However, possible age effects were not considered in this study. Smoking influences these figures, since the overall prevalence of excessive β_2-microglobulin excretion was almost three times higher in smokers than in non-smokers.

Living and farming in areas where Cd pollution by industry is high, as in some regions of Japan, carries a hazard no less than that of direct industrial exposure. Kjellström *et al.* (*ibid* 1977, **13**, 318) examined 138 women aged 51–60 engaged in farming in such a region, where exposure to Cd came mainly from consumption of locally grown rice and river water. The mean urinary Cd level in such people was about twice as high as in controls. Cd concentration in blood and urinary excretion of β_2-microglobulin showed a correlation with the Cd concentration of food rice; β_2-microglobulin excretion also correlated with the period of residence in the polluted area and with the drinking of river water. Kojima *et al.* (*ibid* 1977, **14**, 436) compared 156 farmers living in a Cd-polluted area of Japan with 93 living in a normal area. Since gastro-intestinal absorption of Cd is low (*c.* 5–10%) they used faecal Cd as a measure of Cd exposure and so avoided possible bias both from observers and participants. Mean daily faecal Cd contents were 41 μg in the controls and 146 μg in the exposed group. The mean urinary Cd excretion in the two groups was 2 and 7·5 μg/litre respectively. Urinary excretion of β_2-microglobulin in the controls was 86 μg/litre. Defining tubular proteinuria as the condition when β_2-microglobulin excretion exceeds the mean plus two standard deviations, the prevalence of tubular proteinuria stood at 3% in the controls and 14% in the exposed subjects, and its incidence rose with greater age and longer exposure. Total proteinuria showed a lower ratio of incidence in the two groups than did tubular proteinuria.

Animal studies

In an attempt to elucidate the relationship between Cd-induced bone lesions and kidney lesions, Yoshiki *et al.* (*Archs envir. Hlth* 1975, **30**, 559) fed young rats diets containing 10–300 ppm Cd as $CdCl_2$ for 3 weeks in conjunction with an otherwise normal or a rachitic diet (low calcium (Ca) and no vitamin D). The groups fed 300 ppm Cd continued to be studied for 5–12 weeks. Osteoporotic bone changes particularly affecting the proximal end of the tibia appeared but no osteomalacia, the degree of osteoporosis being related to the Cd intake. These changes persisted until the end of the experiment at 12 weeks. In all groups fed a rachitic diet in addition to Cd the changes were similar to those in the groups fed normal diets with Cd, but were more severe. Metaphyseal trabecula for-

mation was inhibited and epiphyseal cartilage was greatly shortened. In spite of these changes after Cd ingestion for 3 weeks, no pathological changes in the kidney were observed, and there was no detectable urinary excretion of Cd. When the period of feeding of 300 ppm Cd was extended to 5 weeks or longer in conjunction with a normal diet, pathological changes in the kidney became evident at 7 weeks. Atrophic or pyknotic nuclei and cytoplasmic degeneration of a few epithelial cells in limited areas of the proximal tubules were seen. There was some increase in kidney damage by week 12, but most cells remained histologically normal. After Cd treatment for 5 weeks Cd began to undergo urinary excretion at a level of 0·5 μg/ml, increasing to 0·68 μg/ml by week 12. The observations indicate that Cd acts primarily on bone rather than secondarily through inducing kidney dysfunction. However such a short-term study apparently has little relevance to the problems of Cd poisoning in man.

In experiments reported by King *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1976, **16**, 572), male rats received a single sc dose of either 9 or 3 mg Cd/kg, or else were given 0·75 or 0·25 mg Cd/kg three times weekly for 4 weeks. The total doses in the high-dose and the low-dose regimens were about the same for the single or multiple injections. The animals were examined at 4 weeks. In single-dosed animals, concentrations of Cd in the kidney reached higher levels than in the corresponding multiple-dosed animals. In all groups, urinary Cd concentrations remained below 0·05 μg/ml throughout. Weight gain was less in single-dosed than in multiple-dosed rats. In single-dosed rats excretion of urinary protein was greatly reduced, but in multiple-dosed rats it was unaffected. The reduction was attributed to a possible change in testosterone production by Leydig cells of the testes, caused by cadmium accumulation in and around the walls of the capillaries in the testes, or alternatively to a reduced intake of protein. On days 3–21 after a single dose of Cd the urinary concentration of γ-glutamyl transpeptidase significantly decreased; in multiple-dosed rats this effect appeared after day 7. This could have been due to changes in kidney function characteristic of degeneration, or to decreased protein intake or absorption. In the kidney itself there was only a minor change in γ-glutamyl transpeptidase concentration. The evidence indicates minimal destruction of renal tubules by exposure to Cd at the levels used.

Itokawa *et al.* (*Envir. Res.* 1978, **15**, 206) investigated the relationship between Cd toxicity and Ca deficiency. Four groups of five rats were given either a Ca-deficient or a standard diet with or without the addition of 100 ppm Cd, for 60 days. Both control diets contained only 0·03 ppm Cd. In the Cd-fed rats, haematocrit decreased and blood urea nitrogen increased, the latter particularly in the Ca-deficient rats, compared with the controls. Both Cd-fed groups also showed a rise in serum phosphorus (P) although serum levels of Ca were unaffected. There was no significant difference between the groups in urinary protein levels. Both inulin- and P-clearance rates decreased significantly in Cd-fed rats but there was no significant change in the rate of tubular reabsorption of P. The Ca clearance rate decreased and fractional excretion of Ca increased significantly in Cd-fed groups. This suggests that Ca is excreted to a greater extent into the urine when there is renal dysfunction in Cd poisoning. The bone lesions observed may be attributed to this negative Ca balance. The authors cite Washko & Cousins (*Nutr. Rep. Int.* 1975, **11**, 113), who suggested that uptake of Cd into the intestinal mucosa was increased in Ca-deficient rats, a possibility they confirmed in a later study (Washko & Cousins, *J. Toxicol. envir. Hlth* 1976, **1**, 1055). This provides further evidence of the influence of Ca intake on the toxicity of Cd. In their Cd-fed rats, Itokawa *et al.* (*loc. cit.*) found swelling, vacuolar degeneration, and karyorrhexis in the cells of proximal renal tubules. The changes were more prominent in the Ca-deficient rats. There were no histological changes in other parts of the kidney in any group. Granules of Cd were evident in the proximal tubular epithelium of Cd-fed rats.

An experiment of very similar design was carried-out by members of the same group (Kawamura *et al. Nephron* 1978, **20**, 101) using Cd-rich diets containing 50 ppm Cd instead of 100 ppm; the duration of the experiments was increased to 90 days instead of 60 days. Growth of the Cd-poisoned groups was depressed especially in the Ca-deficient group. Total serum-protein and haematocrit levels were lower in rats ingesting Cd. Both inulin and *p*-aminohippuric acid clearance decreased in the Cd-poisoned rats, but the Ca-deficient diet had no influence on renal function. The filtration factor (inulin clearance/*p*-aminohippuric acid clearance) increased in the Cd-poisoned rats. Serum P increased in Cd-fed, Ca-deficient rats. The amount of P excreted decreased in Cd-poisoned rats but fractional excretion of P was unaffected. Serum Ca levels significantly decreased and fractional excretion of Ca showed some increase in Cd-fed rats. In bone (femur), the Ca content decreased and Cd content increased in rats fed Cd; these changes were more prominent in the Ca-deficient animals. Although the increases in Ca and Cd levels in the kidney resulting from the Cd poisoning were not significant, an inverse correlation was found between the Cd content of the kidney and inulin clearance. In some rats of the Ca-deficient, Cd-fed group there was histological evidence of severe renal damage. There was swelling and adhesion between Bowman's capsule and the glomerular tufts, and necrotic and hyalinized glomerular capillaries were evident. Vacuolation, destruction and desquamation were noted in the tubular cells.

It is surprising that both tubular and glomerular lesions were found in these animals, which were exposed to a lower dose of cadmium than that used by Itokawa *et al.* (*loc. cit.*); possibly the longer duration of this experiment was a contributory factor. The localization of kidney damage was not clearly reflected in the clearance studies, although the increased filtration factor and decreased inulin clearance was considered to offer some explanation for the ischaemic changes found in the glomerular capillaries of Ca-deficient Cd-fed rats.

Muto & Omori (*J. Nutr. Sci. Vitaminol.* 1977, **23**, 349) fed young female rats a supplement of 200 ppm Cd, either with a nutritionally deficient or with a balanced diet. Renal abnormalities produced by the high-Cd diet were greatly intensified in the animals

whose diet was deficient in protein, calcium, phosphorus and fibre. The Cd level in the kidneys of the nutritionally-deficient animals was doubled compared with the Cd-fed animals on the balanced diet. It was also found that active transport of ^{45}Ca in the everted gut was decreased in groups of Cd-fed rats. In a subsequent paper, Omori & Muto (*ibid* 1977, **23**, 361) set out to distinguish the roles of the missing nutrients in Cd poisoning. Cd accumulation in the kidney was significantly decreased by the addition of dietary fibre or protein. However the greatest reduction in Cd accumulation was achieved by the addition of Ca and P. Dietary fibre shortened gastro-intestinal transit time, increased faecal output and reduced intestinal Cd levels, indicating that it may inhibit intestinal absorption of Cd. The protective role of protein against renal accumulation of Cd is probably nonspecific and may be associated with general improvements such as increased weight gain and food intake. The dramatic effect of mineral supplementation on suppression of renal Cd levels ties up with the observations of Itokawa *et al.* (*loc. cit.*) on the link between Cd toxicity and Ca deficiency.

Organocadmium

Most of the experimental studies reported have been concerned exclusively with inorganic forms of Cd. Chang & Sprecher (*Envir. Res.* 1976, **12**, 92) found evidence of severe proteinuria after injecting cats five times on alternate days with 3 mg methylCd chloride. In view of the extensive damage produced in such a short time, methylCd seems to be even more nephrotoxic than inorganic Cd. These authors discuss the possibility that Cd, like mercury, could be methylated by micro-organisms and suggest that more attention should be given to the potential hazard from organic Cd.

[P. Cooper—BIBRA]

ANOTHER ROLE FOR LEAD?

The central and the peripheral nervous systems are affected in chronic lead intoxication, and both lead encephalopathy and lead-induced peripheral neuritis occur in man as well as in experimental and other animals (*Cited in F.C.T.* 1975, **13**, 150 & 280). These effects have been recognized for many years, but recently low levels of exposure to lead have been linked with nervous disorders of a more subtle type. It has been suggested that high concentrations of lead in the atmosphere may be responsible for some cases of hyperactivity in children (*ibid* 1975, **13**, 280), and it is also alleged that intelligence may be impaired by early exposure to such atmospheric conditions.

A further suggestion has now developed from a demonstration by Conradi *et al.* (*J. neurol. Sci.* 1976, **29**, 259; *J. Neurol. Neurosurg. Psychiat.* 1978, **41**, 389) of raised levels of lead in the cerebrospinal fluid and plasma of patients suffering from a disorder of the central nervous system known as 'motor neurone disease'. The later of these two communications reports that the plasma-lead concentrations in 16 such patients were significantly higher than those in 18 control subjects, although the actual levels determined by flameless atomic absorption spectrophotometry were lower throughout both groups than the levels generally accepted as 'normal'.

Motor neurone disease is relatively uncommon, with an overall incidence of 2·5–7 cases per 100,000 of the population and the emergence of only about one new case per 100,000 each year (*British Medical Journal* 1978, **2**, 308). It occurs world-wide, however, and in all races, but while its occurrence is generally sporadic, it is endemic in one race living on the island of Guam. This progressive degenerative disorder involves the cranial nerves, the motor cells of the spinal cord and the pyramidal tracts, which are the nerves connecting the brain with the spinal-cord cells. The degeneration of these nerves is reflected in progressive weakness and atrophy of the voluntary muscles, the regions affected depending on the part of the central nervous system involved. Signs of the disease usually develop in people between the ages of 50 and 70 years.

Although it seems likely that the form of the disease found in Guam is genetically determined or dependent on a combination of genetic and environmental influences, the cause and mechanism of motor neurone disease have remained open questions. Following their demonstration of raised plasma-lead levels in patients suffering from the disease, Conradi *et al.* (1978, *loc. cit.*) have postulated that the nerve degeneration could be the result of exposure of the motor end-plates to increased amounts of lead circulating in the plasma with consequent uptake of significant amounts of lead by the nerve cells. They have suggested that the lead is transferred from the end-plates to the body of the cell by a retrograde flow of cytoplasm along the axon. Such a movement was demonstrated by Broadwell & Brightman (*J. comp. Neurol.* 1976, **166**, 257), who identified horseradish peroxidase in the corresponding neurones, following its iv injection close to the motor endplates.

The interesting hypothesis put forward by Conradi *et al.* (1978, *loc. cit.*) will be difficult to investigate experimentally, since lead is less easy to demonstrate in the nerve cells than is horseradish peroxidase, which can readily be identified in tissues by histochemical techniques. Moreover, it must be remembered that, over the years, diverse aetiological factors have been considered in connexion with motor neurone disease. These have included disorders of lipid and carbohydrate metabolism, high concentrations of manganese and calcium in the central nervous system, a blood disease and a defect in the enzyme-producing function of the pancreas. Subsequent investigations have failed to substantiate any of these hypotheses and they have largely been discarded. It may be that the most recent suggestion, implicating prolonged exposure to lead, will suffer a similar fate.

[P. Grasso]

A FLUORIDE BALANCE SHEET

The literature concerning the effects of water fluoridation, the possibilities of fluoride treatment for osteoporosis, and the point at which exposure to fluoride may become hazardous still presents a maze of contradictory assertions and conclusions. Since our last review of available evidence (*Cited in F.C.T.* 1974, **12**, 752), more data on the retention and excretion of ingested fluoride have been obtained from both investigations of human groups and experiments in rats and rabbits.

In three separate studies involving seven patients suffering from osteoporosis (Spencer *et al. Clin. Chem.* 1975, **21**, 613), a diet containing calcium and phosphorus in a ratio of 1:1 and providing (with the drinking-water) an average fluoride intake of 4·36 mg/day was given, and in addition a sodium fluoride supplement supplying a further 20, 40 or 45 mg fluoride daily was given in three divided doses with meals, for mean periods of 129, 90 and 60 days, respectively. Both urinary and faecal excretion of fluoride reflected the size of the supplement, with urinary excretion accounting for 48–63% and faecal excretion for 6–10% of the intake. Faecal excretion was a significant factor in controlling retention of fluoride at the 40 mg/day supplementary level, while urinary excretion, always the main route of fluoride elimination, was particularly effective at the lower level. All the subjects showed a positive fluoride balance, and a marked increase in fluoride retention accompanied any increase in intake. Retention of fluoride continued after supplements had been stopped, and it was assumed, therefore, that retained fluoride was deposited in the skeleton and then firmly incorporated into the crystal structure of the bone.

In another study, Spencer *et al.* (*J. appl. Physiol.* 1975, **38**, 282) determined the urinary and faecal excretion of fluoride in excess of the baseline level when daily fluoride supplements of 9·1 mg given as sodium fluoride for 32 days or of 7·6 mg given as fish-protein concentrate for 26 days were withdrawn from nine patients. The men maintained a low excess excretion of fluoride for 6–12 days after the withdrawal. On average, this excess accounted for 9·1% of retained fluoride given as sodium fluoride and for 14·1% of that given as fish-protein concentrate. Of the excess fluoride, 87% was excreted in the urine and 13% in the faeces.

The effect of oral supplements of calcium (as gluconate) and phosphorus (as glycerophosphate) on eight men taking a diet providing only about 4 mg fluoride daily was studied by the same group (*idem. J. Nutr.* 1975, **105**, 733). The experimental intakes of calcium were 223, 1481 and 2312 mg/day and those of phosphorus were 915 and 1430 mg/day. In a parallel study, the subjects took a diet giving a daily intake of 14 mg fluoride. Neither calcium nor phosphorus supplementation, nor the two together, altered the urinary excretion of fluoride significantly. With both the low- and the high-fluoride diets an increase in calcium intake slightly increased the faecal excretion of fluoride; when a phosphorus supplement was added to a high calcium supplement, faecal fluoride excretion increased by about 50%. Nevertheless, in both treated and control subjects the faecal concentration of fluoride remained low enough for a positive fluoride balance to be maintained.

Hellström & Ericsson (*Scand. J. dent. Res.* 1976, **84**, 187) investigated the effect of fluoridated household salt on ten blind children aged 7–8 years living in an institution and on 34 others aged 2–12 years in normal households. For comparison, nine children in the institution were given 0·5 mg fluoride in tablet form daily for 3 days, and parallel urinary studies were carried out on nine others who had taken 0·25 mg fluoride daily in a regular caries-prevention tablet for 2–6 years. Addition of the fluoridated salt to at least two of the daily meals increased the 24-hour urinary excretion of fluoride from about 0·4 and 0·33 ppm to mean levels of 0·85 and 0·55 ppm in the blind children and in those fed at home, respectively. In previously untreated children given fluoride tablets, the 24-hour urinary excretion of fluoride after ingestion of one tablet was similar to that of children who had received regular daily treatment with 0·25 mg fluoride for a prolonged period. Meals prepared with fluoridated salt provided an intake of 1·5–2 mg fluoride and resulted in a urinary fluoride concentration of up to 3 ppm after 2–4 hours. These Swedish authors suggest that the provision of domestic salt containing 500 ppm fluoride may offer a practical means of caries prevention.

The adverse effects of a very high fluoride intake over a prolonged period came to the fore in a paper by Krishnamachari & Laxmaiah (*Am. J. clin. Nutr.* 1975, **28**, 1234). It has been suggested that the bone deformities, restricted movements of joints and other clinical characteristics of fluorosis are accentuated when diets are inadequate, and particularly when vitamin C intake is low, but these authors failed to demonstrate any change in fluoride excretion as a result of administering vitamin C supplements. Four men who, since birth, had drunk water containing 5·6–8·4 ppm fluoride, were clearly fluorotic, although they showed no obvious effects of nutritional deficiencies. They were all maintained throughout the study on a daily intake of 1–2 mg fluoride, and three of them received 2 g vitamin C daily for 2 weeks while the fourth acted as control. Daily urinary excretion of fluoride varied greatly between subjects but showed less variation in individuals from day to day. Before vitamin C supplementation, the mean 24-hour urinary fluoride outputs for the three subjects were 5·3, 8·5 and 9·7 mg and after the vitamin C treatment the corresponding means were 4·8, 8·5 and 10·8 mg, respectively. The mean values determined for the control subject during these two periods of study were 10·6 and 10·1 mg.

Among the animal studies with a direct bearing on the question of fluoride ingestion and retention is one by Hall *et al.* (*Envir. Res.* 1977, **13**, 285), who treated rabbits by iv injection or intragastric infusion with 0·5 mg fluoride/kg and measured the fluoride concentrations in blood and urine over the following 10 hours. Under steady-state conditions, about 15% of orally administered fluoride was absorbed, entered the extracellular fluid pool, and exchanged with fluoride in the tissues and bone; some 15% of the fluoride intake was then excreted in the urine. The main-

tenance of a steady fluoride balance depended on the existence of a store of fluoride in the gut. Faecal excretion of fluoride amounted to some 85% of the intake. Removal of fluoride from the extracellular pool into the bone compartment was about three times more rapid than its removal into the urine. Some evidence was obtained that the rabbit's response to fluoride is linear over a wide range of doses. In anaesthetized rats, the fractional clearance of fluoride by the kidney was not altered by changes induced in the rate of urinary flow or in fractional chloride clearance by the diuretics frusemide and acetazolamide (Whitford *et al. Am. J. Physiol.* 1976, **230,** 527) but showed a strong dependence on urinary pH. Reabsorption of fluoride was more than 95% complete over the pH range 5·0–5·6, but fluoride clearance rose to 70% when the urinary pH reached 7·8–8·0. There was evidence that the increase in fractional fluoride clearance seen during mannitol-induced diuresis was associated with an increase in urinary pH. It is suggested that while fluoride reabsorption can occur along the entire length of the nephron, it only occurs in the distal part of the tubule under acidic conditions.

In rats maintained on a high fluoride intake (34 ppm fluoride in the food and 50 ppm in the water) for 28 days and then given a low-fluoride diet (0·21 ppm) and distilled water, there was a 12-fold increase in fluoride uptake by the humeri during the 4-week high-intake period, more being taken up by the epiphyses than by the diaphyses (Singer *et al. Proc. Soc. exp. Biol. Med.* 1976, **151,** 627). After the 3-week fluoride-depletion period, however, the skeletal fluoride content had fallen by only 7·7%. These results are not inconsistent with those demonstrated in man by Spencer *et al.* (*J. appl. Physiol.* 1975, **38,** 282). During the period of high-fluoride treatment, there was a significant increase in the fluoride levels of the plasma, muscle and liver, but these concentrations returned to baseline values within 3–7 days of the transfer to the low fluoride intake. These authors report evidence from studies using ionized radioactive fluoride that muscle and liver contain bound fluoride, which is not immediately available for exchange with fluoride ions.

[P. Cooper—BIBRA]

DI-(2-ETHYLHEXYL) PHTHALATE AND THE BLOOD

Significant quantities of di-(2-ethylhexyl) phthalate (DEHP) have been found in blood preparations intended for transfusion and stored in PVC packs (*Cited in F.C.T.* 1971, **9,** 910), and it was demonstrated some years ago that when DEHP migrates from storage bags plasticized with it, the bulk of it becomes concentrated in the plasma-lipoprotein fraction of the stored blood (*ibid* 1973, **11,** 914).

Rubin & Schiffer (*Transfusion* 1976, **16,** 330) have reported on six leukaemic patients who were given platelet infusions from packs made of a vinyl plastics material. Each infusion took 20–30 minutes with the exception of one which lasted for 1 hour. Blood samples were taken before and immediately after the infusion and then at 15-minute intervals for 1 hour. The concentration of the plasticizer in the stored platelets was such that the total DEHP administered to these patients ranged from 26 to 82 mg. Peak plasma concentrations of DEHP, found at the end of the infusion, ranged from 0·34 to 0·83 mg/100 ml and averaged about 0·02 mg/100 ml plasma for each milligram DEHP administered per square metre of body surface. Thereafter the blood concentration of DEHP fell exponentially with a mean disappearance rate of 2·83%/minute, giving a mean half-life of DEHP in blood of 28 minutes. No striking difference in DEHP half-life was apparent in two patients showing mild hepatic-function abnormalities. In two patients whose urinary excretion of DEHP metabolites was studied, 55·9 and 40 mg of DEHP equivalents, or 90 and 60% of the dose, respectively, appeared in the urine in the 24 hours following the infusion. Since, before this platelet infusion, these two patients were excreting 9·0 and 13·8 mg of DEHP equivalents daily (possibly, at least in part, as a result of previous transfusions), the excess excretion of DEHP apparently attributable to the platelet infusion represented about 75 and 40%, respectively, of the administered plasticizer.

Relatively rapid clearance and excretion of DEHP was thus demonstrated in these patients, and the wide use of preparations of this type as well as the results of animal testing have so far provided little evidence of adverse effects. Nevertheless it is important to remember that patients given blood transfusions may be suffering from a wide variety of abnormalities and may be more susceptible than healthy people to the effects of foreign chemicals. The situation is further complicated by the fact that the pattern of tissue distribution has been shown to vary with the physical state of the DEHP. Rubin & Schulz (*Toxic. appl. Pharmac.* 1974, **29,** 143) demonstrated, for example, that the physical state of the ester in iv-administered solutions greatly affects the rapidity of its clearance from plasma and the extent of its retention in the liver. While present evidence indicates that the DEHP present in vinyl-stored blood products is in a solubilized state rather than in the form of suspended oil droplets (Rubin & Schiffer, *loc. cit.*), this situation is not reflected in much of the experimental toxicity and distribution data available. This is because the plasticizer's low solubility in water has led to the use of solubilizing agents or physical techniques, such as sonication, in the preparation of test solutions. Solubilization with Tween 80, which has been used in some studies, has been shown to cause a marked increase in the acute toxicity of iv doses of DEHP (Schulz, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1974, **33,** 234).

To avoid such complications, Waddell *et al.* (*Toxic. appl. Pharmac.* 1977, **39,** 339) incorporated into an

autoradiography study a procedure designed to provide a test solution containing DEHP in the physical state in which it would be likely to be encountered in blood stored in plastics bags. By immersing DEHP labelled with ^{14}C in the carbonyl group in sterile mouse plasma, for 78 days at 22°C, they achieved a concentration of 2·293 mg DEHP/ml. Intravenous injections of 1 ml of this plasma were given to adult male mice, which were anaesthetized and frozen for whole-body autoradiography after 1, 3, 9 or 24 hours or 7 days. Others, injected with the labelled DEHP 30 minutes after ligation of both renal pedicles, were subjected to autoradiography 1 or 3 hours after the injection. The responses of germ-free mice to these treatments were compared with those of conventional animals. In all the mice there was rapid accumulation of radioactive label in the kidney and liver, but not in other organs. This was demonstrated 1 hour after the injection, but by 3 hours considerable elimination into the urine, bile and intestine had occurred and elimination was virtually complete within 9 hours, except in the mice with ligated kidneys. These showed a more prolonged retention of radioactivity and a more scattered distribution in the blood and extracellular fluids.

Attachment of DEHP to protein constituents of human plasma, with physical alteration, has been demonstrated by Stern *et al.* (*ibid* 1977, **41,** 507), in further attempts to define the form of the DEHP actually encountered by patients given blood transfusions from PVC packs. In human plasma stored at room temperature for 10 days in PVC packs plasticized with [^{14}C]DEHP, they demonstrated that DEHP formed a non-particulate diffusion complex and that the extent of migration was determined not by the plasma lipid content but by the total protein concentration. In non-particulate solutions prepared by the careful addition of ethanolic DEHP to plasma, a large proportion of the DEHP was shown to be bound to the α_2- and β-globulins. When the diffusion complex of [^{14}C]DEHP was injected into the tail vein of rats, the second phase of ^{14}C half-life in the blood was shorter than when a complex formed from an ethanolic or Polysorbate-80 solution was used, organ retention was reduced and excretion was more rapid, at least initially. When included in the perfusing fluid for an isolated rat-liver preparation, the protein diffusion complex of DEHP was metabolized and rapidly excreted in the bile.

These findings provide further evidence that the physico-chemical reactions of DEHP with plasma are complex, and that when toxicity studies of this plasticizer are undertaken in connexion with its presence in transfused blood, the non-particulate complex, which is the actual blood contaminant, should be used, rather than other solubilized or particulate forms.

[P. Cooper—BIBRA]

EVIDENCE OF A SAFE LEVEL OF TDI

Toxicology cannot class toluene diisocyanate (TDI) as one of its success stories. In spite of considerable scientific interest, the mechanism underlying its toxic action still remains elusive (*Cited in F.C.T.* 1972, **10,** 730; *ibid* 1976, **14,** 218; *ibid* 1977, **15,** 365). The inability of the toxicologist to define an acceptable atmospheric concentration of TDI is a more practical disappointment.

Among recent additions to the TDI literature is a report by Wegman *et al.* (*Br. J. ind. Med.* 1977, **34,** 196) on a follow-up of an earlier investigation of workers involved in the production of polyurethane. The original study (*idem, J. occup. Med.* 1974, **16,** 258) demonstrated that exposure to TDI resulted in an acute reduction in pulmonary function. The same workers were re-examined 2 years later to assess the chronic effects of TDI. Only 63 of the 112 workers originally studied were still employed and therefore available for examination, but the pulmonary function of the retested and lost groups of workers was comparable in 1972 and there was little evidence of a selection bias in the restudied group.

TDI levels at the workplace were determined five times during the 2 years between the two investigations and were found to be stable. The workers were divided into three groups of approximately equal size: those exposed to TDI levels in excess of 0·0035 ppm, those exposed to between 0·002 and 0·003 ppm TDI, and those exposed to levels below 0·0015 ppm. Although the incidence of subjective symptoms of pulmonary disorder was similar in all three groups, the prevalence of coughing and phlegm increased with increasing exposure and there were variations in performance in pulmonary-function tests. The group exposed to the lowest levels of TDI had a 2-year change in forced expiratory volume (FEV) within the expected limits for a normal population, but in the other two groups, loss in FEV increased with increasing exposure. Even when all possible contributory factors were considered, including age, smoking habits and lung size, the differences between the groups were still significant, and it was concluded that a loss of pulmonary function occurred at TDI exposures of 0·0035 ppm with a possibility that exposures as low as 0·002 ppm were having some effect.

Wegman *et al.* (1977, *loc. cit.*) stressed the importance of measuring the factory levels of TDI as well as length of work service in any prospective study of the effects of this particular monomer. If length of service had been adopted as the sole index of exposure, no significant correlation would have been found between exposure and reduction in FEV. These authors also noted the possible complications introduced by the well-documented acute effects of TDI; at high exposures FEV decreases over every work-shift, whereas at lower levels, acute loss occurs on the first day of a working week. Because of this, it was emphasized that lung function should be exam-

ined immediately before commencement of work after at least a weekend away from the plant, to provide baseline measurements.

The results presented in this study confirmed earlier indications (*Cited in F.C.T.* 1969, 7, 699) of a correlation between acute and chronic loss of FEV. It was suggested that workers who exhibit large acute pulmonary changes following TDI exposure should be frequently re-examined so that chronic changes in lung function may be detected at an early stage.

In another study, the health of a number of workers in a TDI-manufacturing plant was monitored by Butcher *et al.* (*Am. Rev. resp. Dis.* 1977, **116**, 411) over a 2·5-year period. For purposes of comparison, the workers were divided essentially into three groups: those who had daily contact with TDI (group I), maintenance personnel who had intermittent contact (group II), and a group of non-exposed controls. All groups were comparable in atopic status, age, sex and race but were not matched for size or smoking habits. Before the start of TDI production (April 1973), and at roughly 6-monthly intervals, the immunological status and health of the workforce was evaluated.

Of the 103 workers interviewed in April 1973, 89 subsequently undertook jobs entailing regular or intermittent exposure to TDI. Over the period of the study, the continuously monitored levels of TDI in the factory air often exceeded the present TLV of 0·02 ppm, the maximum transient level being 0·04 ppm. In the exposed group there was a significant increase ($P < 0{\cdot}01$) in the prevalence of lower respiratory problems (cough, wheezing, shortness of breath), compared with the control group. In other respects, including the incidence of bronchitis and upper respiratory symptoms, immunoglobulin levels, peripheral-blood eosinophil counts and the average performance in pulmonary-function tests, TDI exposure had no discernible adverse effects. Unfortunately the results of the pulmonary-function tests were analysed only with respect to length of TDI exposure which, in the light of the conclusions drawn by Wegman *et al.* (1977, *loc. cit.*) may explain the apparent absence of a TDI-related effect.

In spite of these largely negative results, a significant proportion of the workforce studied by Butcher *et al.* (*loc. cit.*) did develop sensitivity to TDI. For example, 13 workers demonstrated clinical sensitivity to the monomer and, of these, nine suffered an adverse bronchial response when challenged with TDI exposures from 0·005 to 0·02 ppm. Even in these sensitive individuals, the controlled TDI exposure did not result in any increase in the blood eosinophil count. Inhalation challenge with acetyl-β-methylcholine generated a bronchial response in eight of the 11 TDI-sensitive individuals who were tested, whereas only one out of ten non-sensitized subjects gave a similar positive response. This may have predictive value in the screening of workers for bronchial hyperreactivity before they enter jobs involving potential TDI exposure.

An increasing incidence of TDI-specific IgE antibodies was detected using a radioallergosorbent (RAST) test. The first positive results, a single case in each of groups I and II, were found in March 1975. Although by April 1976 eight workers exposed to the TDI gave evidence of allergenicity, two workers of the control group were similarly affected. There was also an increase in the number of people who gave a positive response in a skin test with TDI-human serum albumin (HSA) conjugate; a total of ten individuals of either group I or II exhibited positive but small reactions during the 3 years of observation, the first positive result occurring in March 1974. Somewhat surprisingly none of the workers who gave evidence of sensitivity to TDI in the RAST or patch tests were among the thirteen subjects who demonstrated clinical sensitivity to TDI.

Wegman *et al.* (1977, *loc. cit.*) mentioned their use of personal as well as area sampling and drew attention to an earlier study in which this precaution had not been taken. Comparative air-sampling studies carried out by Butcher *et al.* (*loc. cit.*) confirmed the need to relate observed biological effects with individual levels of exposure rather than with levels deduced from area monitoring, by demonstrating a marked lack of correlation between the results of personal and area measurements. The monitoring programme used by Wegman *et al.* (1977, *loc. cit.*) does not seem entirely satisfactory, however, and it would be of value if their data could be confirmed and extended by a study involving the regular measurement of breathing-zone concentrations of TDI at intervals considerably more frequent than the five measurements in 2 years used as the basis for their reported study.

In addition to providing further clues to safe exposure levels of TDI, these reports underline some of the pitfalls involved in such studies and some of the precautions that must be observed if meaningful results are to be obtained not only for TDI but also for other industrial chemicals.

[J. Hopkins—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

Absorption of polymeric food colourings

Honohan, T., Enderlin, F. E., Ryerson, B. A. & Parkinson, T. M. (1977). Intestinal absorption of polymeric derivatives of the food dyes sunset yellow and tartrazine in rats. *Xenobiotica* **7**, 765.

Water-soluble azo dyes such as tartrazine and sunset yellow are cleaved at the azo bond by gut bacteria (*Cited in F.C.T.* 1969, **7**, 87). The products of tartrazine fission are sulphanilic acid (SA) and 1-(4-sulphophenyl)-3-carboxy-4-amino-5-pyrazolone (SCAP), which may be further metabolized to SA, whereas sunset yellow produces SA and 1-amino-2-naphthol-6-sulphonic acid (ANSA) (*ibid* 1963, **1**, 101; Roxon *et al. Fd Cosmet. Toxicol.* 1967, **5**, 447). Ingestion of tartrazine may provoke allergic reactions such as asthma and urticaria (*ibid* 1973, **11**, 685), and the FDA has proposed that it should be declared by name on food products (*Food Chemical News* 1976, **18**(47), 29). Sunset yellow has also been implicated in occasional cases of hypersensitivity (Baer & Leider, *J. invest. Derm.* 1949, **13**, 223; Michaëlsson & Juhlin, *Br. J. Derm.* 1973, **88**, 525). In the latter study, SA failed to produce urticaria or other objective signs of allergy in eight subjects sensitive to sunset yellow and tartrazine.

Polymeric antioxidants which are absorbed to only a very limited extent from the gut have recently been developed (*Cited in F.C.T.* 1977, **15**, 357; *ibid* 1978, **16**, 321) and parallel work is in hand on colourings (*ibid* 1978, **16**, 321). The metabolic fate of polymeric derivatives of tartrazine and sunset yellow is compared with that of the free colourings in the paper by Honohan *et al.* cited above. In both cases the polymer was a polysulphanilamidoethylene (PSAE), a polyaminoethylene backbone being linked through a sulphonamide group to the azo dye.

When rats were given, by gavage, the polymeric derivative of tartrazine (PSAE-TA), uniformly labelled with ^{14}C in the 1-*p*-sulphophenyl ring, 4·6% of the ^{14}C appeared in the urine. Over half of this was identified as SCAP and about 40% as SA. After administration of similarly labelled tartrazine, urinary radioactivity accounted for almost the same proportion (4·0%) of the dose, 44% being SCAP in this case. SA represented a further 43% of the excreted radioactivity. Thus the amount of SA derived from the aminopyrazolone moiety was approximately the same for both forms of tartrazine; in the case of PSAE-TA, it was equal to about 0·9% of the total equivalents administered. After oral administration of ^{14}C-labelled PSAE itself, a negligible proportion of the ^{14}C was excreted in the urine in 72 hr, whereas after oral administration of ^{14}C-labelled SCAP, 8·9% of the radioactivity appeared in the urine, of which 45% was unchanged SCAP and 52% was SA. This indicated that the urinary SA in rats given PSAE-TA was derived from further metabolism of SCAP, rather than directly from cleavage of the azo and sulphonamide bonds. From colorimetric determination of total (labelled and unlabelled) urinary SA, which was equivalent to over 20% of the dose following administration of tartrazine itself, it was calculated that use of the polymeric form of the dye reduced absorption by about 95%. No unchanged tartrazine was found in the urine after dosing with either tartrazine or PSAE-TA.

When the ^{14}C-labelled polymer derivative of sunset yellow (PSAE-SY) was administered orally, the urine contained 6·9% of the administered radioactivity, which in the absence of detectable unchanged sunset yellow or SA was assumed to be ANSA. In rats given ^{14}C-labelled sunset yellow, total urinary activity represented 8·5% of the dose. Intact dye accounted for 1–2%, and the remainder was again assumed to be ANSA. Urinary excretion of SA was equivalent to 37–40% of the dose after administration of either labelled or unlabelled sunset yellow. About 1·5% of the unchanged dye was also excreted in the bile, but excretion of PSAE-SY in the bile was not examined.

The study indicated that azo-bond cleavage occurred as readily in the polymer derivatives PSAE-TA and PSAE-SY as in the dyes themselves but the resulting SA moiety remained, in both cases, attached to the polymer backbone. The authors suggest that the decrease in absorption of unchanged dye and of the SA produced by azo reduction resulting from the use of polymeric derivatives may be significant in developing non-sensitizing substitutes for tartrazine and sunset yellow.

[This is an interesting development, which is still at a relatively early stage of investigation. The resistance of the polymers to absorption from the gut and the apparent absence of any significant retention of the dyes or their metabolites in the body will need further confirmation. Further work will also be needed to establish conclusively the chemical structures responsible for the cases of human hypersensitivity associated with these colourings.]

FLAVOURINGS, SOLVENTS AND SWEETENERS

Mutations from saccharin

Batzinger, R. P., Ou, S.-Y. L. & Bueding, E. (1977). Saccharin and other sweetners: Mutagenic properties. *Science, N.Y.* **198**, 944.

Whether saccharin is or is not a bladder carcinogen is a subject of continuing controversy. Mutagenicity studies have also presented problems of interpretation and a review of 17 such studies concluded that they had produced only conflicting or equivocal results

(Kramers, *Mutation Res.* 1975, **32,** 81). Now, however, comes another report of mutagenicity, both *in vivo* and *in vitro*.

The saccharin preparations tested consisted of both a retail (A) and a wholesale (B) pharmaceutical material, a sample (C) from the lot used in a Canadian rat-feeding study in which bladder tumours were found (*Food Chemical News* 1977, **19**(15) 39), a highly purified version (D) of the last type, a powder (E) containing 40 g saccharin and 1 g lactose-potassium tartrate filler in a widely used convenience package, and a second sample (F) from a different lot used in the Canadian study. Samples E and F were used in the *in vitro* studies only, but the sweeteners xylitol and neohesperidin were included in all the tests. The test materials (2·5 g/kg) were given to mice by stomach tube and mutagenicity was determined with *Salmonella typhimurium* strains TA98 and TA100 (used respectively to detect frameshift mutations and base-pair substitutions). These bacterial strains were incubated either with the 24-hr urine or in the peritoneal cavity of the mice (host-mediated assay), and they were also used to detect mutagenicity *in vitro*, using sweetener concentrations about 20% below those producing 10% growth inhibition.

In the urine assay, saccharin samples A–C were mutagenic to both strains, and activity towards strain TA98 was markedly enhanced after incubation of the urine with β-glucuronidase. The addition of a rat-liver microsomal preparation (S_9) increased mutagenicity to this strain but inhibited activity towards strain TA100, suggesting that two different mutagens were present. The urines of mice given sample D demonstrated low but significant activity to strain TA100; this was increased by S_9 after β-glucuronidase treatment. The excreted product(s) of this highly purified saccharin showed no mutagenic activity against TA98, however.

In the host-mediated assay, samples A–C displayed a lower activity, and D was inactive. Similarly, of the saccharin samples tested *in vitro*, only D failed to display any significant activity, the other saccharin samples being weakly mutagenic to both strains. The activities of samples A and B were increased by S_9 but those of C and E were not, while sample F was activated by S_9 in tests with TA100 but not in those with TA98. No mutagenicity was demonstrated with xylitol or NHDC under any conditions.

PRESERVATIVES

Nitrited pork for rats and mice

Knudsen, I. & Meyer, O. A. (1977). Mutagenicity studies on rats and mice given canned, heated, nitrite-treated pork. *Mutation Res.* **56,** 177.

The possible induction of human cancer by consumption of nitrosamines formed from food amines and the nitrite used in curing and preserving food has received a great deal of attention (*Cited in F.C.T.* 1978, **16,** 69 & 389). The study reported below examines the possibility of a dose relationship between nitrite added to pork and genetic damage in germ cells, leading to dominant lethality, sterility or semi-sterility in the F_1 generation.

Lean salted pork was treated before canning and heating with 200, 1000 or 4000 ppm sodium nitrite and fed to rats and mice as their sole protein source throughout the period of spermatogenesis (to rats for 12 wk and to mice for 8 wk). Just after the end of treatment the males were allowed to mate (three virgin females were introduced each week) over a period of 3 wk for mice and 5 wk for rats. The females were killed and dissected on day 15 (mice) and day 17 (rats) after introduction to the males. At no feed level of nitrited pork did treated rats differ from the controls in pregnancy rate, number of implantation sites or percentage of dead implantations. In mice fed pork treated with 4000 ppm nitrite there was a significant decrease in the number of implantations from the wk 3 matings, but no associated increase in post-implantation loss. The F_1 males for the heritable translocation test were derived from male and female mice both given nitrite-treated pork, at the levels indicated above. Females mated to the F_1 males were killed and dissected 15 days after introduction to the males. Fertility was considered to be normal if two out of three females mated to the same male had ten or more implants and the average live litter size for the three females was at least eight. The appearance of one semi-sterile male among 50 in the maximally exposed group of mice in the heritable translocation test could not be regarded as significant; nor could the one completely sterile mouse found among the 90 fed pork containing 1000 ppm nitrite in the dominant lethal test. Experiments with more animals are needed to reach a firmer conclusion in the heritable translocation test. The absence of any dose-related response in dead implantations in the dominant lethal test indicates that the levels of sodium nitrite used in the treated pork do not increase dominant lethal mutations.

Nitrosamines in saliva

Tannenbaum, S. R., Archer, M. C., Wishnok, J. S. & Bishop, W. W. (1978). Nitrosamine formation in human saliva. *J. natn. Cancer Inst.* **60,** 251.

The role of nitrosamines in carcinogenesis and their possible formation in the human gut from the reaction between amines in foods and added nitrites were recently reviewed (Cooper, *Fd Cosmet. Toxicol.* 1978, **16,** 69). Nitrites are also found in normal human saliva, probably as a product of microbial reduction of nitrate, which circulates through the salivary glands. The study cited above examined the nitrosation reaction in saliva and in saliva mixed with acid to simulate conditions in the stomach.

Human saliva in 5-ml aliquots was incubated with an amine and in some cases with additional nitrite for 30 min at 37°C. Resulting nitrosamine concentrations were determined using gas chromatography

with a thermal energy analyser. In whole saliva from several individuals a low but consistent amount of nitrosamine, around 30 nmol/litre, was synthesized in all samples tested, even in saliva containing relatively low nitrite concentrations. Saliva from one individual was used to determine the effects on nitrosamine formation of adding 1000 mg morpholine/litre (a) alone, (b) with 100 mg sodium nitrite/litre or (c) with HCl to achieve a pH of 3. Yields of nitrosomorpholine (NMOR) were determined after incubating whole saliva (containing living cells), cell-free supernatant, resuspended cells, heated whole saliva and a buffer control under these three regimes. After incubation with morpholine alone, NMOR was detected (12 nmol/litre) only with unheated whole saliva. When sodium nitrite was also added to the incubation mixture, NMOR was found in all five systems ranging from 188 nmol/litre with the resuspended cells to 5 nmol/litre with the buffer control. Whole saliva produced about a third as much NMOR as did the resuspended cells and very little was produced by either the cell-free supernatant or the heated whole saliva. At the lowered pH, whole saliva, cell-free supernatant and heated whole saliva produced respectively 3176, 1676 and 1857 nmol NMOR/litre. No NMOR was produced by the acidified resuspended cells or the buffer control. Significant amounts of nitrosamines were also formed on addition of di-*n*-propylamine, di-*n*-butylamine and di-*n*-hexylamine to whole saliva, while addition to dimethylamine, diethylamine, piperidine and pyrrolidine produced trace amounts.

The results indicate that nitrosamine formation is possible in saliva even at neutral pH. Cells in whole saliva accelerate nitrosation, although the saliva contains components that both accelerate and retard nitrosamine formation. The investigators also showed that various amines may be nitrosated in saliva and that the rate of the reaction was independent of the basicity of the amine but dependent on the length of the alkyl chain. The results support their hypotheses that (1) nitrosamine formation in the mouth is catalysed by the oral microflora, (2) nitrite formed in the oral cavity contributes to nitrosamine formation in the normal stomach, (3) nitrosamines can be formed in the weakly acidic or even neutral stomachs of patients with intestinal metaplasia and (4) nitrosamines can be formed at other sites that normally contain bacteria or become infected.

[It seems surprising that the authors used saliva from one individual for the bulk of this experiment, especially since the saliva chosen had a much lower nitrite concentration (2·5 mg/litre) than the saliva samples used in the preliminary experiment (mean, 7·0 mg nitrite/litre).]

AGRICULTURAL CHEMICALS

Dieldrin, DMSO and the pregnant mouse

Dix, K. M., van der Pauw, C. L. & McCarthy, W. V. (1977). Toxicity studies with dieldrin: teratological studies in mice dosed orally with HEOD. *Teratology* **16,** 57.

Cleft palate and webbed foot have been described in the offspring of mice and hamsters exposed to dieldrin during pregnancy, but these effects may have been relegated to inhibition of growth (*Cited in F.C.T.* 1975, **13,** 397) and the design of the study excluded any possibility of detecting a dose–response relationship. The mouse study now reported was carried out on 1,8,9,10,11,11-hexachloro-4,5-*exo*-epoxy-2,3,7,6-*endo*-2,1,7,8-*exo*-tetracyclo[$6.2.1.1^{3,6}.O^{2,7}$]dodec-9-ene (HEOD), which constitutes about 85% of dieldrin. The HEOD used was over 99% pure.

Female mice were dosed orally with 1·5 or 4·0 mg HEOD/kg/day in corn oil from day 6 to day 14 of gestation and were examined on day 18. Additional groups received 0·25, 0·5 or 1·0 mg/kg/day in dimethylsulphoxide (DMSO) for the same period. Some mice given HEOD in DMSO, or DMSO alone, became dyspnoeic and hypothermic and showed decreased motor activity. One animal given DMSO, one given 0·25 mg HEOD/kg in DMSO, and four given 1·0 mg/kg in DMSO died during the study. Significantly fewer mice survived to term in the groups given 0·5 or 1·0 mg/kg in DMSO than in the other groups, but none of the treated groups showed consistent differences in the mean numbers of male and female foetuses in the litters, mean litter size, numbers of resorptions or numbers of foetal deaths. In the DMSO study, the test groups had foetuses of significantly lower weight than the controls, but this did not occur in the corn oil study. Most of the skeletal abnormalities observed in both experiments involved minor changes in the sternum. None of the treated groups showed any significant increase in the number of litters containing foetuses with structural abnormalities, but delayed ossification showed a higher incidence in litters of the group given 0·5 mg HEOD/kg in DMSO and of that given DMSO alone. These two groups also showed a significant increase in the proportion of total foetuses showing minor skeletal changes.

These results provide no evidence of any compound-related teratogenic effects and suggest that the effects observed were largely a reflection of the toxicity of the DMSO used as a vehicle in the second experiment.

Liver nodules in mirex study

Ulland, B. M., Page, N. P., Squire, R. A., Weisburger, E. K. & Cypher, R. L. (1977). A carcinogenicity assay of Mirex in Charles River CD rats. *J. natn. Cancer Inst.* **58,** 133.

Reuber, M. D. (1977). Acute and chronic renal hepatic disease in rats fed Mirex. *J. natn. Cancer Inst.* **59,** 1051.

Ulland, B. M. (1977). [Reply to M. D. Reuber.] *J. natn. Cancer Inst.* **59,** 1052.

Mirex (dodecachloro-octahydro-1,3,4-metheno-2*H*-cyclobuta[*c*,*d*]-pentalene) has been used in the place of the more persistent dieldrin and heptachlor to control the red fire ant. It has been shown to produce liver damage and cataract in rats (*Cited in F.C.T.* 1971, **9**, 590), and when fed to mice has induced tumours (*ibid* 1970, **8**, 229).

In the initial study quoted above, 26 rats of each sex were fed 50 or 100 ppm mirex for 18 months and observed for a further 6 months, in conjunction with positive controls (20 of each sex) fed 80 or 250 ppm *N*-2-fluorenylacetamide (2-AAF). The groups showed no major differences in weight gain. Untreated controls survived longer than mirex-treated animals taking the larger dose. All groups showed a high incidence of a wide variety of neoplasms, 50% of males and 80–88% of females being affected.

The livers of significant numbers of males receiving 100 ppm mirex showed neoplastic nodules, which apparently progressed to hepatocellular carcinomas in some animals. Unlike the tumours induced by 2-AAF, these did not metastasize to the lung. Neither neoplastic nodules nor carcinomas developed in the livers of untreated controls, although several control animals showed foci or areas of cellular alteration.

The criticism offered by Reuber (*loc. cit.*) is based on his own interpretation of the slides from the mirex study. He points out that severe liver necrosis or fatty change, necrosis and/or degeneration of renal tubular cells, mild to severe nephritis and myocarditis were common findings in the treated rats, and that such toxic effects may have influenced the incidence and development of neoplastic lesions. In reply, Ulland agrees that the toxic and life-shortening effects of mirex may influence tumour incidence but states that the incidence of cardiomyopathy in males and females and of nephropathy in males did not appear to be related to the feeding regime.

Liver carcinomas from heptachlor

Reuber, M. D. (1977). Histopathology of carcinomas of the liver in mice ingesting heptachlor or heptachlor epoxide. *Expl Cell Biol.* **45**, 147.

A recent National Cancer Institute study (NCI Technical Background Information: Report on Carcinogenesis Bioassay of Chlordane and Heptachlor; September 1977) confirmed earlier indications that heptachlor could produce liver carcinomas when fed to mice (BIBRA Bull. 1977, **16**, 443). One of the earlier studies, conducted by O. G. Fitzhugh and K. J. Davis and reported briefly to the FDA in 1965, involved feeding C3H mice with a 10 ppm dietary level of heptachlor or heptachlor epoxide from the age of 3 wk for up to 24 months. The histopathology of the carcinomas that were found in these mice is now reported in detail.

Liver carcinomas were found in 73% of males and 74% of females ingesting heptachlor, and in 92% of males and 95% of females ingesting heptachlor epoxide. By contrast, only 30% of male controls and 4% of female controls developed such tumours. The livers of treated mice often contained more than three or four carcinomas, which were predominantly hepatocellular and ranged from undifferentiated to well-differentiated. The latter grew in cords, with focal canaliculi and sinusoids containing lining cells, and the cells had a vesicular nucleus with prominent nucleoli and abundant eosinophilic cytoplasm. Undifferentiated and poorly differentiated carcinomas grew in sheets, with much variation in the size and shape of nuclei and cells and basophilic cytoplasm. Vacuoles containing water or lipid were often present in the carcinoma cells. The carcinomas invaded hepatic veins, and lung metastases were found in a number of cases. Hepatic vein thrombosis also occurred in 10·5% of treated mice.

More on MCPA toxicity

Hattula, M. L., Elo, H., Reunanen, H., Arstila, A. U. & Sorvari, T. E. (1977). Acute and subchronic toxicity of 2-methyl-4-chlorophenoxyacetic acid (MCPA) in male rat. I. Light microscopy and tissue concentrations of MCPA. *Bull. env. contam. & Toxicol.* (*U.S.*) **18**, 152.

Elo, H. & Parvinen, M. (1976). Effect of sodium 2-methyl-4-chlorophenoxyacetate on spermatogenesis in the rat. *J. Reprod. Fert.* **48**, 243.

MCPA (2-methyl-4-chlorophenoxyacetic acid) is widely used as a contact herbicide. Its short-term toxicology has been described (Gurd *et al. Fd Cosmet. Toxicol.* 1965, **3**, 883; *Cited in F.C.T.* 1976, **14**, 214).

In the first study cited, male rats were given sc injections of 300–700 mg sodium MCPA/kg and the LD_{50} was calculated as 500 mg/kg, all doses within the experimental range producing myotonia within 15–30 min, with diarrhoea. Autopsy findings included extensive haemorrhages in the gastro-intestinal and urinary tracts, and the most obvious histopathological changes were in the liver and spleen. The liver sinusoids were hyperaemic and there were necrotic foci in the parenchymal cells, particularly near portal areas. The lesions were most marked after the 700-mg/kg dose. Spleen changes were mainly in the white pulp, which showed necrotic foci. No notable changes were observed in organs other than the liver and spleen. Tissue concentrations of MCPA ranged from 120 to 480 μg/g wet tissue, and were consistently highest in kidney tissue.

In a subacute experiment, rats were given 100–2000 mg sodium MCPA/litre of drinking-water for 9 wk. With concentrations of 1000 mg/litre or more, the animals drank less water, weight gain was retarded and the relative weights of liver, brain and adrenals were increased. At 2000 or 3000 mg/litre the relative weight of the kidney was also increased. No gross pathological changes were seen but the histological changes resembled those reported for the acute experiment.

In the second study cited, mild changes in the seminiferous epithelium of some rats followed the consumption of water containing 100–3000 mg sodium MCPA/litre. With 1000 mg/litre or more, weight gain was again retarded but the relative weight of the testes was not altered. In the seminiferous epithelium, spermatid maturation stages 15–19 were lost in some tubules. This loss was enhanced when the concentration reached 2000 mg/litre and was

sometimes accompanied by degeneration of young spermatids and pachytene spermatocytes. In some damaged tubules, young round nucleated spermatids formed giant cells. However, some animals consuming water containing 3000 mg/litre retained almost normal spermatogenesis.

Tissue distribution of ethylenethiuram monosulphide

Iverson, F., Newsome, W. H. & Hierlihy, S. L. (1977). Tissue distribution of ethylenethiuram monosulfide (ETM) in the rat. *Bull. env. contam. & Toxicol. (U.S.)* **18**, 541.

The ethylenebisdithiocarbamate fungicides are readily degraded by plant and animal systems to yield ethylene thiourea (ETU) and ethylenethiuram monosulphide (ETM). Investigations have suggested that ETU is responsible for the tumorigenic properties of the parent compounds (Graham *et al. Fd Cosmet. Toxicol.* 1975, **13**, 493), whilst more recent studies (*Cited in F.C.T.* 1977, **16**, 361) have examined the teratogenicity of ETU and related compounds, including ETM. Of the compounds tested in this latter study, only 4-methylethylenethiourea produced anomalies that were indistinguishable in type and frequency from those produced by ETU. ETM is considered to be the principal fungitoxic oxidation product of this group of pesticides, although there is little information available on the toxicity of the compound to animals. The above-cited study was therefore undertaken to examine the tissue distribution of ETM as well as to provide a comparison of ETU with ETM under similar experimental conditions.

An acute oral toxicity study was carried out in the rat. Tissue distribution studies were then undertaken following ip and oral administration of ETU and ETM. For ip administration to rats, ^{35}S-labelled ETU (17·6 μCi/mg) was prepared in distilled water and administered at 4 mg/kg body weight (1·0 ml/kg). [^{14}C]ETM (11·29 μCi/mg) in corn oil was administered at 5 mg/kg (1·0 ml/kg). Rats were killed at intervals between 0·5 and 24 hr to provide samples of blood, heart, lung, thyroid, liver, kidney, spleen, fat and muscle (and brain for ETM). Thyroid samples were also taken after longer periods following ETM treatment. For oral dosing, rats were dosed each day for 6 days with [^{35}S]ETU (5·0 ml/kg body weight; 4 mg/kg) in distilled water or with [^{14}C]ETM (5·0 ml/kg; 5 mg/kg) in corn oil. Duplicate samples of tail blood were taken 24 hr after each dose and samples of blood, heart, lung, liver, kidney, spleen, fat, muscle and thyroid were taken at autopsy 24 hr after the final dose, for determinations of total radioactivity.

The 24-hr LD_{50} for ETM was 240 mg/kg. ETM exhibited a biphasic decline in organ radioactivity whilst with ETU the decline was linear. All tissues revealed a consistent decline in radioactivity with the exception of the thyroid. The thyroid radioactivity after repetitive oral ETM dosing (6 days) was about 5 times higher than that occurring 24 hr after a single ip dose, suggesting the possibility of accumulation. However, thyroid radioactivity following repeated ETU dosing was only about 1·5 times higher than that demonstrated 24 hr after a single ip dose. The investigators postulate that this situation might arise from a saturation mechanism. A steady state was approached within the 6-day dosing regimen, as indicated by a sharp increase in radioactivity after the first dose of ETU or ETM and then a gradual increase over the remaining dosing period. The absence of intact ETM from the urine and the low adipose-tissue levels of radioactivity after administration of [^{14}C]ETM, indicate that ETM has a relatively short half-life *in vivo*. Results of the study also suggest that ETU could arise *in vivo* after ingestion of ETM residues, although the lack of ETU metabolism was not consistent with the results obtained after ETM administration. A dose-dependent metabolism of ETU might explain this discrepancy and further studies are in progress to define more closely the metabolism and excretion of ETM and ETU.

Tumours from succinic acid 2,2-dimethylhydrazide

Toth, B., Wallcave, L., Patil, K., Schmeltz, I. & Hoffmann, D. (1977). Induction of tumours in mice with the herbicide succinic acid 2,2-dimethylhydrazide. *Cancer Res.* **37**, 3497.

The carcinogenicity of hydrazine and a number of its derivatives has been established in animals (*Cited in F.C.T.* 1973, **11**, 897) and succinic acid 2,2-dimethylhydrazide (SADH) has now been added to the list of derivatives showing evidence of carcinogenic potential. This compound is a herbicide and plant-growth regulator, reported to be used in substantial quantities in fruit cultivation in some areas.

From the age of 6 wk until death, equal numbers of male and female mice were given a 2% solution of SADH as their sole source of drinking-water. This concentration, which was chosen on the basis of a short-term study, provided an average daily intake of 134 mg SADH for females and 170 mg for males. Survival time was significantly shortened by SADH, the last treated mouse dying before wk 100, compared with wk 130 in controls. At autopsy, tumours of the blood vessels, lungs and kidneys, respectively, were found in 73, 73 and 5% of SADH-treated mice but in only 6, 18 and 0% of controls. The blood-vessel tumours were chiefly angiosarcomas, with some angiomas, and occurred principally in the liver. The lungs contained both adenomas and adenocarcinomas, while the kidney tumours (which were found only in males) were all adenomas. A few tumours of other types were observed, but they were rare and could not be attributed to SADH treatment. It was thought possible that hydrolysis to 1,1-dimethylhydrazine may have accounted to some extent for the carcinogenic effect, but as commercial SADH is also dissolved in water the same situation would apply.

Chlorodibenzo-*p*-dioxins as teratogens

Courtney, K. D. (1976). Mouse teratology studies with chlorodibenzo-p-dioxins. *Bull. env. contam. & Toxicol. (U.S.)* **16**, 674.

We have recently reviewed the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ('dioxin') which has

gained notoriety recently as an environmental hazard (*Cited in F.C.T.* 1977, **15**, 481). The high toxicity of dioxin has prompted studies of the toxicity of other chlorinated dibenzo-*p*-dioxins, using female CD-1 mice.

The compounds studied were dibenzo-*p*-dioxin (I), 2,7-dichlorodibenzo-*p*-dioxin (II), a 40/60 mixture of II with 2,3,7-trichlorodibenzo-*p*-dioxin (III), 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (IV), octachlorodibenzo-*p*-dioxin (V), and dioxin itself. Oral doses ranging from 5 μg/kg to 50 mg/kg, depending upon the compound, and given daily for 14 days demonstrated no lethal dose. Dioxin was the most toxic of the compounds in the acute experiments, and I, II and V were relatively non-toxic. Oral doses of III at 100 and 200 μg/kg/day given on days 7–16 of pregnancy did not adversely affect maternal or foetal parameters; in marked contrast, dioxin at 100 μg/kg/day reduced foetal weight and survival and maternal weight gain, and higher doses produced generalized maternal oedema and vaginal bleeding, which was sometimes followed by abortion, after the sixth dose. Dioxin was even more lethal to the foetus when injected sc. At an oral dose of 5 mg/kg/day, V did not affect either mother or foetus adversely, and the reduction in foetal weight associated with 20 mg/kg/day was not statistically significant. Treatment with III was associated with a slight increase in the incidence of abnormal foetuses, but analysis of this increase in terms of dose and individual litters suggested that it was of doubtful significance. Compound IV had no effect on the incidence of foetal malformations. Dioxin itself was teratogenic at all dose levels studied, when administered by the oral or sc routes, the most common abnormalities being cleft palate and hydronephrotic kidneys. The incidence of abnormal foetuses per litter was about 87% with the lowest dose of dioxin (25 μg/kg), so a dose-related response could not be demonstrated.

PROCESSING AND PACKAGING CONTAMINANTS

The hazards of chewing PVC

Casterline, C. L., Casterline, P. F. & Jaques, D. A. (1977). Squamous cell carcinoma of the buccal mucosa associated with chronic oral polyvinyl chloride exposure. *Cancer, N.Y.* **39**, 1686.

Whilst vinyl chloride is an established carcinogen, its carcinogenic activity in man has so far been demonstrated only in workers who were involved in the polymerization industry and were exposed to high atmospheric concentrations of the vapour. The current debate centres on the significance of very small levels of the monomer in food. An interesting, although somewhat peripheral addition to the bibliography of vinyl chloride has now been reported.

The paper concerns the case of a man, aged only 22, who developed an invasive squamous-cell carcinoma of the buccal mucosa. This is a rare type of tumour, since although about 5% of all tumours are classified in the USA as squamous-cell carcinomas of the head and neck, only some 1% of these involve the buccal mucosa. Oral cancers are almost invariably associated with heavy smoking or drinking, or arise from chronic irritation associated with faulty dentition, but in this specific instance, the patient neither smoked nor drank alcohol. He did indulge, however, and had done for over 14 years, in the unusual habit of chewing plastics materials. These materials, typically PVC-coated wire insulation, were often stored in the mouth for periods up to 8 hr. Although there was no evidence of faulty dentition, his teeth were observed to be grooved due to his habit of using them for stripping wire!

The authors considered that the man's unusual habit was, in all probability, directly responsible for the unusual pathology observed, and in support of this hypothesis cited the results of a recent mortality study of workers involved in the manufacture of vinyl chloride and its polymers. These workers showed an increased incidence of buccal and pharyngeal cancers, although within the group the occurrence was inversely proportional to vinyl chloride exposure (Tabershaw & Gaffey, *J. occup. Med.* 1974, **16**, 509). In this connexion, it was suggested that the mouth and pharynx might be particularly susceptibile because of the gaseous nature of the exposure.

[An hypothesis derived from a single case history must be considered tentative in the extreme. A more fitting conclusion to the present report might well be "there's nowt so queer as folk".]

THE CHEMICAL ENVIRONMENT

Toluene has a brain wave

Takeuchi, Y. & Hisanaga, N. (1977). The neurotoxicity of toluene: EEG changes in rats exposed to various concentrations. *Br. J. ind. Med.* **34**, 314.

Many workers exposed to organic solvents such as toluene develop non-specific disturbances of the central and autonomic nervous systems including headache, heaviness of the head, giddiness, forgetfulness, insomnia, fatigue, lassitude, loss of appetite, nausea, palpitation and impotence. Although abnormal electroencephalograms (EEGs) in toluene workers have been reported (Mabuchi *et al. Igaku no Ayumi* 1974, **88**, 97; Takeuchi *et al. Jap. J. ind. Hlth* 1972, **14**, 563), few attempts have been made to clarify experimentally the relationship between cause and effect.

The studies reported in the above cited paper were designed to study the changes in EEG and behaviour of rats following exposure to various concentrations of toluene vapour.

Chronically implanted electrodes were used in male Wistar rats to record EEGs for the cortex and hippocampus, the cervical electromyogram (EMG) and the pulse rate. Groups of four or five animals were exposed to 4000, 2000 or 1000 ppm toluene vapour for 4 hr during daylight because the rat, being a nocturnal animal, tends to sleep during the day. The sleep cycle was divided into five phases with regard to the EEG, EMG of cervical muscles and behaviour of the animals (wakeful, spindle, slow-wave, pre-paradoxical and paradoxical).

In animals exposed to 4000 ppm toluene, all of the sleep phases were drastically reduced and rarely occurred during the 4 hr of exposure or the first 2 hr after exposure had ended. In the 2000-ppm group, the wakeful phase increased significantly, and the spindle, slow-wave, pre-paradoxical and paradoxical phases all decreased significantly on the exposure day in comparison with those observed on the preceding day. In the group exposed to 1000 ppm, the pre-paradoxical phase decreased significantly and the spindle phase increased significantly on the exposure day in comparison with the preceding day. The paradoxical phase tended to increase during all 6 hr, and this increase differed significantly during the first 2 hr after the end of exposure from that noted on the preceding day. Changes in the EEG components were peculiar to each concentration. Dose-related behavioural changes and increases in the pulse rate were observed after exposure to the compound.

The investigators consider that the changes in basic EEG rhythms are significant findings which are detectable in both humans and animals exposed to high concentrations of toluene vapour. They suggest that further studies involving prolonged periods of exposure are desirable.

[The changes recorded in these animals are hardly surprising, since the rats in the 4000-ppm group were unable to walk or crawl during the latter half of the exposure time, and those in the 2000-ppm group became unsteady on their legs towards the end of exposure. More relevant results could perhaps be obtained by repeated exposure of animals to toluene at concentrations closer to the present TLV of 200 ppm.]

Zinc chromate as a lung carcinogen

Davies, J. M. (1978). Lung-cancer mortality of workers making chrome pigments. *Lancet* **I**, 384.

It has been reported that the incidence of bronchial cancer shows an increase among workers making chromate pigments, particularly zinc chromate (*Cited in F.C.T.* 1976, **14**, 215).

A study of 396 men who started work in one factory between 1932 and 1967, 136 who began work at another between 1948 and 1967, and 114 who started work at a third between 1946 and 1967 offers further evidence of this association between lung cancer and exposure to zinc chromate. Data on men who died up to mid-1977 showed that in the first two factories there was an excess of deaths from lung cancer; the manufacture of zinc chromate had ceased in the first in 1964 and in the second in 1976, but lead chromate pigments are still made in both. There was no increase in deaths from lung cancer in the third factory, manufacturing lead chromate pigments only, suggesting an association between the manufacture of zinc chromate and the increase found in the other two factories. Mortality figures were similar after exposure to high levels of dry and dusty pigments and after exposure to medium levels of wet and less dusty pigments, and showed a ratio of observed to expected deaths of 9 to 2·97 after exposure for as little as 1 yr. Men who entered employment at Factory A after 1954, when working conditions were improved, showed no increase in lung cancer deaths. When mortality data were analysed for 5-yr intervals after the start of work, induction times were found to be relatively short and most deaths from lung cancer occurred in ex-workers not more than 25 yr after their first exposure to zinc chromate. Nevertheless most of the deaths occurred after the workers had ceased to be employed at the factories, so the hazard remained inconspicuous.

Hexachlorobutadiene and the kidney

Kociba, R. J., Keyes, D. G., Jersey, G. C., Ballard, J. J., Dittenber, D. A., Quast, J. F., Wade, C. E., Humiston, C. G. & Schwetz, B. A. (1977). Results of a two year chronic toxicity study with hexachlorobutadiene in rats. *Am. ind. Hyg. Ass. J.* **38**, 589.

Damage to the kidney tubular epithelium was the chief pathological finding in rats made to inhale hexachlorobutadiene (HCBD) concentrations of 25 or 100 ppm for 6 hr/day for 12 or 15 days (Gage, *Br. J. ind. Med.* 1970, **27**, 1). The kidney also appeared to be the most sensitive organ in an unpublished study conducted by Dow Chemical U.S.A., in which rats were fed 1–100 mg HCBD/kg/day for 30 days. Renal tubular degeneration and necrosis were found at 30 mg/kg and above, with minimal hepatocellular swelling at 100 mg/kg and haemoconcentration at 10 mg/kg and above.

In the Dow Chemical study now reported, rats were fed 0·2, 2·0 or 20·0 mg HCBD/kg/day for up to 2 yr. At 20 mg/kg weight gain was depressed, as was the red blood cell count in males, and urinary coproporphyrin excretion was increased in both sexes. There was a significant increase in male deaths during the last 2 months. Relative and absolute kidney weights were increased in males, while in females there was a significant increase in relative weights of brain and kidney, and a decrease in absolute liver and heart weights. Renal tubular adenomas and adenocarcinomas were found in nine of 39 males and six of 40 females, and were accompanied by hyperplasia and focal adenomatous proliferation of the renal tubular epithelium. By contrast, such tumours were found in only one of 180 controls. Two of the kidney tumours metastasized to the lungs. There was also a decrease in subcutaneous neoplasms (mainly of mammary origin) and an increase in adenomatous uterine polyps, but an abnormally low incidence of these polyps in the controls, rather than the ingestion

of HCBD, seemed the most likely reason for this apparent increase.

In rats fed 2·0 mg HCBD/kg, urinary coproporphyrin excretion was significantly increased in females after 14 months. There was also a possible increase in hyperplasia of the renal tubular epithelium, and adenomatous proliferation of the epithelium was evident in females, but no increase in tumours of the kidney or other organs was found. At 0·2 mg/kg, no changes were evident which could be attributed to treatment.

TRIS and human DNA

Gutter, B. & Rosenkranz, H. S. (1977). The flame retardant *tris*(2,3-dibromopropyl)phosphate: alteration of human cellular DNA. *Mutation Res.* **56,** 89.

The flame retardant tris-(2,3-dibromopropyl) phosphate (TRIS) has now been subjected to a wide range of toxicological studies. Primarily as a result of its carcinogenic effects in experimental animals, its use was banned in the USA (*Federal Register* 1977, **42,** 18850), and restrictions have also been placed on it in other countries. TRIS has proved to be mutagenic in bacterial systems (Prival *et al. Science, N.Y.* 1977, **195,** 76) and the authors cited above examined the effects of the compound on human cellular DNA.

Monolayers of growing KB cells were grown in the presence of [^{3}H]thymidine for 30 hr, washed free of the labelled compound and then grown for a further 17 hr in fresh medium to exhaust the [^{3}H]-labelled soluble pool. The cells were then exposed for 4·5 hr either to TRIS or to medium of TRIS. They were processed for analysis of the DNA on alkaline-sucrose gradients either immediately after the TRIS and control treatments or after the monolayers had been washed twice with complete medium and re-incubated for various intervals to permit DNA repair.

Exposure of growing cells to TRIS resulted in a reproducible decrease in the size of the DNA molecule, as indicated by a decreased rate of sedimentation in gradients of alkaline sucrose. After incubation in the absence of TRIS, the DNA of treated cells sedimented in the same region of the gradient as did the DNA derived from untreated cells. This evidence of DNA repair indicates that the decreased size of the DNA from treated cells was not a reflection of a non-specific toxic effect (e.g. autolysis), since in that case the DNA would not have been repaired. Rather it suggests that TRIS may have genetic effects in eukaryotic cells as well as in bacteria.

Another dampener on flame retardants

Loewengart, G. & Van Duuren, B. L. (1977). Evaluation of chemical flame retardants for carcinogenic potential. *J. Toxicol. envir. Hlth* **2,** 539.

The loss of 'TRIS' from the flame-retardants market (*Federal Register* 1977, **42,** 18850) was followed by a switch to suitable alternative compounds. However, these other retardants are now being subjected to the same degree of toxicological attention as was afforded to TRIS, an examination that could possibly lead to the similar disappearance of some of them. This study investigates the carcinogenic potential of tetrakis(hydroxymethyl)phosphonium chloride (THPC) and the related compound Pyroset TKP, which are widely used in flame-retardant fabrics and particularly in children's sleepwear.

THPC is synthesized from phosphine, hydrochloric acid and formaldehyde and decomposes to these chemicals as a result of both thermal and chemical treatments. It is possible that the carcinogen bis-chloromethyl ether (BCME) is formed either as a by-product during THPC synthesis or through an equilibrium reaction, giving rise to a potential hazard in the synthesis, industrial use and end-product use of THPC and THPC-treated fabrics. In addition, solutions such as sweat, urine and saliva may extract free THPC, thus subjecting a proportion of the child population to long-term low-level exposure to the compound. In order to examine these possibilities, extraction studies were undertaken on cloth treated with THPC, in addition to mouse skin-painting studies of the two flame retardants.

Although the compounds necessary for the formation of BCME were all present in large amounts in aqueous THPC solutions, BCME was not detected in concentrations above 0·1 ppm. Formaldehyde analyses demonstrated that the amount of phosphonium ion extracted from treated cloth was similar whether the extractant was a synthetic sweat solution or distilled water, and elemental analysis of a lyophilized extract gave 14·7% P and 10·8% N, confirming that some form of the THPC finish was being released from the fabric into the aqueous medium. Both the chemical nature and toxicological status of this extracted material at present remain uncertain. In the mouse skin-painting studies, THPC showed weak carcinogenic potential but moderate tumour-promoting activity (following initiation with 7,12-dimethyl-[*a*]anthracene) and Pyroset TKP showed moderate tumour-promoting activity. The tumour yield and multiplicity were low but many of the tumours progressed to squamous carcinomas. However the effect of the DMSO vehicle on the positive controls complicated the estimation of the promoting activity of the two flame retardants.

[Despite the findings of the carcinogenicity studies, further work is required to identify the components of the extracts and evaluate their toxicological status.]

1,1,2-Trichloroethane under the skin

Kronevi, T., Wahlberg, J. & Holmberg, B. (1977). Morphological lesions in guinea pigs during skin exposure to 1,1,2-trichloroethane. *Acta pharmac. tox.* **41,** 298.

Jakobson, I., Holmberg, B. & Wahlberg, J. E. (1977). Variations in the blood concentration of 1,1,2-trichloroethane by percutaneous absorption and other routes of administration in the guinea pig. *Acta pharmac. tox.* **41,** 497.

The solvent 1,1,2-trichloroethane (vinyl trichloride: TCE) is used as a raw material for vinylidene chloride synthesis. It has shown a degree of hepatotoxicity between that of chloroform and that of trichloroethylene (*Cited in F.C.T.* 1975, **13,** 588) and studies of its percutaneous toxicity are therefore important.

In the first study cited above, it was found that, in pentobarbitone-anaesthetized guinea-pigs, direct exposure of the dorsal skin to TCE for periods ranging from 15 min to 12 hr produced morphological changes in the epidermis. These were evident after 15 min and progressed with longer exposure. Pyknotic nuclei appeared, with perinuclear oedema of the basal and suprabasilar cells and focal separation of the epidermis from the corium by vesiculation. In animals exposed to TCE for 6 hr, the glycogen content of the liver was reduced and there were hydropic changes in the centrilobular areas. After a 12-hr exposure, these liver changes had become less marked. They did not occur at all in animals that were not anaesthetized for the experiment, a finding consistent with an earlier report (Carlson, *Life Sci.* 1973, **13,** 67) that another barbiturate, phenobarbitone, potentiated the hepatotoxic effect of TCE in rats, when administered some time before the solvent. The kidney and brain showed no changes.

In the second study cited, guinea-pigs anaesthetized with pentobarbitone were treated either with 1 ml TCE applied to the skin or with 50 μl given in a single intracutaneous, sc or ip injection. TCE was detected in the blood within 5 min of the epicutaneous application; its concentration increased over 30 min, and then fell over 1 hr, subsequently increasing again gradually over the following 4 hr. A second epicutaneous application at another site was followed by a new rise in blood concentration of TCE, a second minimum, and then a gradual increase. After ip injection, the blood concentration of TCE rose rapidly to a peak at 2 hr, declining over the next 10 hr. After intracutaneous or sc injection TCE was taken up more gradually into the blood and then slowly declined. The complex toxicokinetics of TCE absorbed percutaneously are apparently due to a local skin effect rather than to a systemic response and may be complicated by progressive skin damage, which alters the barrier function.

Dangerous car repairs

Clausen, J. & Rastogi, S. C. (1977). Heavy metal pollution among autoworkers. I. Lead. *Br. J. ind. Med.* **34,** 208.

Clausen, J. & Rastogi, S. C. (1977). Heavy metal pollution among autoworkers. II. Cadmium, chromium, copper, manganese, and nickel. *Br. J. ind. Med.* **34,** 216.

Car repair workshops are a potential source of hazardous materials, particularly toxic metals. The preliminary results of a Danish study of workers in this industry suggest that further detailed investigation is warranted.

Blood samples were taken from 216 individuals employed in the car repair industry and from 54 subjects of a comparable age who had no professional involvement in the accumulator, garage or metal industry. The average concentration of lead in the blood of all the car workers was $44{\cdot}8 \pm 22{\cdot}8\ \mu g/100$ ml, a level significantly higher than the $18{\cdot}6 \pm 9{\cdot}6\ \mu g/100$ ml measured in the controls. These elevated lead levels were associated with a corresponding decrease in the blood δ-aminolaevulinic acid dehydratase activity. Although the average cadmium, copper and manganese blood levels were comparable in the exposed and control groups, the car workers had significantly higher concentrations of chromium and nickel in their blood. There was no correlation between the individual levels of lead, nickel and chromium, and presumably, therefore, there was no common source of these metals. However there was a slight correlation between copper and nickel levels.

Mean atmospheric concentrations of lead in the car workshops were $3{\cdot}19 \pm 2{\cdot}29\ \mu g/m^3$. Theoretical calculations showed that 8-hr daily exposures to this concentration could not have been totally responsible for the observed level of lead in the blood. Chromium and nickel were present in the air only at levels below their respective threshold limit values; again inhalation alone could not explain the high levels in the blood, and absorption through the skin was probably a significant route of exposure for all the heavy metals. One sample of unused gear oil, for example, was shown to contain 9280 ppm lead and used car oils contained up to 3438 ppm lead, up to 27 ppm chromium and up to 30 ppm nickel. Almost a third of the mechanics, who accounted for over half of the car workers in the study, suffered from various skin complaints, which may have facilitated skin absorption.

Carboxyhaemoglobin levels in excess of 12% were found in 19% of the car workers and 71% of this group complained of dizziness and headache. A previous study of garage workers (Buchwald, *Am. ind. Hyg. Ass. J.* 1969, **30,** 570) also produced evidence of a work-related increase in the blood level of carboxyhaemoglobin.

NATURAL PRODUCTS

3-Methylindole invades the lungs

Huang, T. W., Carlson, J. R., Bray, T. M. & Bradley, B. J. (1977). 3-Methylindole-induced pulmonary injury in goats. *Am. J. Path.* **87,** 647.

3-Methylindole (skatole; 3MI) is one of a number of metabolites produced by enteric bacterial flora and it has been suggested that this 'faecal toxin' is harmful to human tissues, although the target tissues and the nature of injury remain unknown. 3MI has been shown to give rise to pulmonary oedema in cattle and goats (Carlson *et al. Science, N.Y.* 1972, **176,** 298). As cigarette smoke contains substantial quantities of 3MI, indole and other neutral indole derivatives, an examination of the nature and pathogenesis of 3MI-induced lung injury was made with the aim of shedding light on the pathogenesis of lung diseases associated with cigarette smoking.

The 3MI was administered to nine goats orally in gelatin capsules at a dosage of 0·16 g/kg body weight

and pairs of the animals were killed after 2, 4, 8 and 24 hr, the survivor being killed at 48 hr. Blood was collected at 30-min intervals initially and later every 2 hr, and lung-tissue samples were taken at autopsy from the treated goats and from three untreated controls.

There was a steady rise in plasma 3MI concentration and peaks were reached between 1 and 2 hr after treatment, returning to zero after 12 and 15 hr respectively, in the two goats examined beyond 8 hr. The animals developed pulmonary oedema and laboured breathing at 4 hr and this became increasingly severe up to 15 hr. One animal with a peak 3MI concentration of 13·8 μg/ml died at 8 hr with severe dyspnoea. Electron-microscope studies of the lungs revealed extensive degeneration and necrosis of alveolar membranous pneumocytes and bronchiolar epithelium. Necrosis of the pneumocytes was followed by a repair process involving proliferation of granular pneumocytes. 3MI also induced proliferation of the smooth endoplasmic reticulum in the remaining membranous pneumocytes and non-ciliated columnar epithelium of the small airways, indicating that these two types of cell are involved in the foreign-compound metabolism effected by the lung.

The results suggest that cigarette smoke, which may contain 0·4–1·4 mg 3MI/100 cigarettes as a product of tryptophan pyrolysis, could play an important role in the pathogenesis of small airway disease and emphysema. In addition, patients with a severe liver disease or portacaval shunt may be predisposed to diffuse alveolar damage by 3MI produced in the intestinal tract.

Patulin ingestion by monkeys

Garza, H. C., Swanson, B. G. & Branen, A. L. (1977). Toxicological study of patulin in monkeys. *J. Fd Sci.* **42**, 1229.

Patulin is a heterocyclic lactone produced by various species of Penicillium and Aspergillus, which may contaminate bread and pastry, fruits and cereals. It has been reported to inhibit lactic dehydrogenase, alcohol dehydrogenase and muscle aldolase (Ashoor & Chu, *Fd Cosmet. Toxicol.* 1973, **11**, 617 & 995; *Cited in F.C.T.* 1977, **15**, 495).

Pig-tail monkeys (*Macaca nemestrina*) were given daily oral doses of 5, 50 or 500 μg patulin/kg for 4 wk. Those given 500 μg/kg were subsequently given 5 mg/kg/day for a further 2 wk. Blood samples were taken weekly for haematology and biochemical determinations. The only statistically significant effect of patulin was a decrease in serum alkaline phosphatase, a finding probably meaningless in isolation. Serum proteins showed their normal electrophoretic patterns, and blood counts were unaltered. There was a slight increase in blood urea nitrogen during wk 2 and 3, but this was of no clear biochemical significance. The monkeys showed no signs of toxicity during the 4-wk treatment period or in the extended high-dose period that followed. Those transferred to the 5-mg/kg/day dose rejected the freeze-dried bananas that were used throughout the experiment for administration of the patulin, presumably either because they found the flavour of the lactone or the citrate buffer unpleasant or because these flavours had become associated with stress. The low toxicity of patulin by the oral route, compared with its effects when given parenterally, is possibly due to the rapid absorption and metabolism of the toxin, and to its prompt excretion.

Tree pollution?

Boyd, M. R., Statham, C. N., Franklin, R. B. & Mitchell, J. R. (1978). Pulmonary bronchiolar alkylation and necrosis by 3-methylfuran, a naturally occurring potential atmospheric contaminant. *Nature, Lond.* **272**, 270.

3-Methylfuran (3MF) was found to be a major contaminant of the smog that occurred in Washington, DC, during the summer of 1973. As it has been postulated that 3MF is a photo-oxidation and degradation product of terpenes and other volatile hydrocarbons released in large amounts by vegetation such as deciduous forest, this compound may be a common environmental factor. The results of a preliminary study of the inhalation toxicity of 3MF have now been reported.

A high degree of covalent binding of [*Me*-^{3}H]3MF to the microsomes of mouse lung occurred *in vitro*. As the binding reaction required an NADPH-generating system and was inhibited by an atmosphere enriched with carbon monoxide or by addition of piperonyl butoxide to the medium, it appeared that the lung microsomes were converting 3MF to an alkylating metabolite by means of a cytochrome *P*-450-dependent oxidation. A similar metabolic activation occurred *in vivo*. If mice were exposed to atmospheric concentrations of 0·23 mmol [^{3}H]3MF/litre for 1 hr, the 3MF became covalently bound to the lung macromolecules and induced severe bronchial necrosis. Histologically it was shown that the metabolites accumulated preferentially in the terminal pulmonary bronchioles. Both the degree of covalent binding and the extent of bronchial necrosis was markedly reduced in mice pretreated with an ip injection of piperonyl butoxide (1·6 g/kg).

The observed effects were produced by high concentrations of 3MF. Nevertheless, these preliminary findings, incriminating 3MF as a possible cause of bronchiolar disease in man and raising the question of its possible carcinogenic potential, suggest that further investigation is warranted.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

Chlorhexidine—percutaneous penetration

Chow, C. P., Buttar, H. S. & Downie, R. H. (1978). Percutaneous absorption of chlorhexidine in rats. *Toxicology Lett.* **1**, 213.

Chlorhexidine is now widely used in place of hexachlorophene as an antimicrobial agent in cosmetics and toiletries. In this application it produces only rare cases of sensitization (*Cited in F.C.T.* 1974, **12**, 799),

although eye irritation may result from its use as a soft-lens sterilant (*ibid* 1975, **13**, 288). Most of an oral dose administered to a number of species was excreted in the faeces, and (except in the mouse) less than 1% appeared in the urine, while after iv injection some 50–70% appeared in the bile within 48 hr (*ibid* 1974, **12**, 587). Its fate after skin application has now been similarly quantified.

When 296 μg ^{14}C-labelled chlorhexidine in aqueous acetone was applied to shaved rat skin under a non-occlusive plastics guard, a mean total of 1·1% of the radioactivity had appeared in the faeces after 5 days. The urine contained a total of only 0·07%, and the carcass (after excision of the application site) a further 3·2%. The skin application site contained approximately 98% on average. After iv injection of the same quantity in saline some 80% had been excreted in the faeces and 3·3% in the urine after 5 days, and the carcass contained a residual 22·5%. When the ratio of urinary excretion after topical and after iv application was expressed as a percentage, to give an estimate of the total percutaneous absorption (a method recommended by Feldmann & Maibach, *J. invest. Derm.* 1970, **54**, 399) a value of 2·0% was reached. The total absorbed through the skin in 5 days was thus in the range 2·0–4·3%. This compared with a value of 76·6% from an equimolar dose of hexachlorophene administered in the same way (Chow *et al. Toxicology* 1978, **9**, 147), a difference which could be attributed to the greater lipid-solubility and lower molecular weight of the latter compound.

Decline and fall of a sensitizer

Smith, S. Z. & Epstein, J. H. (1977). Photocontact dermatitis to halogenated salicylanilides and related compounds. *Archs Derm.* **113**, 1372.

The halogenated salicylanilides and related compounds have been widely used as antibacterial agents in soaps, cleansers and medicated toilet preparations. However, over the period 1960–1970, many cases of photosensitization to these materials were reported (*Cited in F.C.T.* 1969, **7**, 690). In the USA, from where many of the reports emanated, bithionol was officially excluded from incorporation into cosmetic and antibacterial preparations in June 1968. Subsequently, the dibrominated compounds (1972) and tribomosalicylanilide (1974) were eliminated from such use. The above-cited study examines experience with these compounds at the University of California's San Francisco clinic over the period June 1967 to October 1975.

The subjects involved were 434 patients who had either a history suggestive of a photosensitivity problem or an eruption involving sun-exposed areas at least in part. Age, sex and racial distribution were tabulated and the numbers of patients with positive photopatch tests, positive patch tests and completely negative tests were compared. The majority of patients tested and the majority of patients with positive photopatch tests were in their fifth to sixth decade. None were children, and men were more commonly involved than women, the ratio being approximately 8:5. No racial trends could be detected in those tested or in those with positive phototests.

Over the period of study there was a distinct decline in the numbers of patients with positive patch and photopatch test reactions and in the number tested. Almost three times as many were tested and four times as many had positive photopatch tests in the last 6 months of 1967 as in the first 9 months of 1975. There were also 16 positive non-irradiated patch tests in the 1967 period and only one in 1975. The most striking reduction of patients with positive tests occured in 1971, although the total number of positive photopatch tests dropped most notably after 1968. In 1967 the compounds that elicited the greatest number of positive photopatch tests were 3,5-dibromosalicylanilide, 3,4′,5-tribromosalicylanilide, bithionol, 3,3′,4′,5-tetrachlorosalicylanilide and polybromosalicylanilide. The general reduction after this date resulted from a lower incidence of reactivity to these brominated salicylanilides and to 3,3′,4′,5-tetrachlorosalicylanilides.

Although the primary reason for the decline was probably the removal of the more potent photosensitizers from deodorant soaps, it is also believed that a greater awareness among physicians played a role in reducing referrals to the clinic.

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MEETING ANNOUNCEMENTS

METABOLIC EFFECTS OF ALCOHOL

An International Symposium on the Metabolic Effects of Alcohol will be held in Milan on 18–21 June 1979. The symposium will be promoted by the Centro Studi dell'Alimentazione/Nutrition Foundation of Italy, the Institute of Pharmacology and Pharmacognosy of the University of Milan and the National Institute on Alcohol Abuse and Alcoholism, USA. The preliminary programme includes the following topics: alcohol, health and society; interactions with drugs; proteins, carbohydrates and collagen, lipids and lipoproteins; the endocrine system; effects on liver function and pathology; direct cardiovascular and renal effects. The working languages will be English and Italian, and simultaneous translation will be provided. Details and application forms may be obtained from the Organizing Secretariat, Centro Studi dell'Alimentazione, Nutrition Foundation of Italy, Via S. Pietro all'Orto 17, 20121 Milan, Italy.

RECLAMATION OF CONTAMINATED LAND CONFERENCE

The Society of Chemical Industry has announced a Conference on the Reclamation of Contaminated Land, to be held in Eastbourne on 22–25 October 1979. The conference aims to identify the problems presented by the re-use of land previously contaminated, to consider the contributions of the various disciplines and technologies involved, and to recommend further study and action. Further information may be obtained from The Conference Secretariat, Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS.

CUTANEOUS TOXICITY

The Fourth Conference on Cutaneous Toxicity sponsored by the American Medical Association and the Society of Toxicology will be held at the Mayflower Hotel, Washington, DC, 9–11 May 1979. The registration fee is $150 ($120 for AMA and SOT members; $85 for residents and retired physicians). For further information contact Dr. Joseph B. Jerome, American Medical Association, 535 North Dearborn Street, Chicago, IL 60601.

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FORTHCOMING PAPERS

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

2-Bromo-2-nitropropane-1,3-diol as a nitrosating agent for diethanolamine: a model study. By I. Schmeltz and A. Wenger.

In vitro metabolism of [^{14}C]rubratoxin B by rat hepatic subcellular fractions. By P. D. Unger, M. Y. Siraj and A. W. Hayes.

The determination of respirable particles in talcum powder. By R. S. Russell, R. D. Merz, W. T. Sherman and J. N. Sivertson.

Urinary silicon excretion by rats following oral administration of silicon compounds. By G. M. Benke and T. W. Osborn.

Studies on the action of histamine release by persulphates. By J. F. Parsons, B. F. J. Goodwin and R. J. Safford.

Comparisons of response of Fischer-344 and Charles River rats to 1·5% nitrilotriacetic acid and 2% trisodium nitrilothriacetate, monohydrate. By R. L. Anderson and R. L. Kanerva.

Detection of mutagenic polycyclic aromatic hydrocarbons in African smoked fish. By K. Mossanda, F Poncelet, A. Fouassin and M. Mercier. (Short Paper)

Inhibitory action of the mycotoxins patulin and penicillic acid on urease. By J. Reiss. (Short Paper)

Mycophenolic acid in marketed cheeses. By P. Lafont, M. G. Siriwardana, I. Combemale and J. Lafont. (Short Paper)

The 'carry-over' of aflatoxin, ochratoxin and zearalenone from naturally contaminated feed to tissues, urine and milk of dairy cows. By B. J. Shreeve, D. S. P. Patterson and B. A. Roberts. (Short Paper)

Application of the diffusion theory to migration of plastics components into packed goods: survey of recent migration studies. By C. G. vom Bruck, F. B. Rudolph, K. Figge and W. R. Eckert. (Review Paper)

Cancer, mathematical models and aflatoxin. By F. W. Carlborg. (Review Paper)

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Physiopathology of haemorrhagic syndrome related to ochratoxin A intoxication in rats (*P. Galtier, B. Boneu, J. L. Charpenteau, G. Bodin, M. Alvinerie and J. Moré*) 49

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Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Some other Pergamon Journals which may interest readers of *Food and Cosmetics Toxicology:*

Annals of Occupational Hygiene

European Journal of Cancer

Archives of Oral Biology

Health Physics

Atmospheric Environment

Journal of Aerosol Science

Biochemical Pharmacology

Journal of Neurochemistry

Chronic Diseases

Toxicon

Life Sciences

Each journal has an individual Information and Index Leaflet giving full details. Write now for any leaflet that interests you.

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

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In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. **1.** Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

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

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

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