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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

CHRONIC TOXICITY OF BUTYLATED HYDROXYTOLUENE IN WISTAR RATS

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(*Received 28 March 1980*)

Abstract—Groups of 57 Wistar rats of each sex were maintained on diet containing 0·25 or 1% butylated hydroxytoluene (BHT) for 104 wk; control groups comprised 36 rats of each sex. Treated rats of both sexes showed reduced body-weight gain, relative spleen weight and white-blood-cell count while in the males there was also a reduction in serum triglyceride. BHT-treated animals of both sexes showed increased relative liver weight and total blood cholesterol but increases in red-blood-cell count could be seen only in females and only the males showed increased γ-glutamyltransferase. No significant histological changes were observed in the liver or haematopoietic system to explain these haematological and biochemical changes. Tumours were found in the liver, pancreas, mammary glands, uterus, pituitary gland, adrenal glands and in some other organs of some of the treated rats, but their incidence was not significantly different from that in controls. This experiment showed no carcinogenic effect of BHT on rats.

INTRODUCTION

Butylated hydroxytoluene (BHT) is an antioxidant widely used in foods, cosmetics and pharmaceutical products to prevent oxidation of labile lipid components. It has been shown to have antimutagenic activity in the *Salmonella typhimurium* reversion test (McKee & Tometsko, 1979) and tests *in vivo* showed that it induced injury followed by cell proliferation in the lungs of mice (Adamson, Bowden, Cote & Witschi, 1977), and severe nephropathy in both Wistar and SD rats (Meyer, Blom & Olson, 1978). It has also been found to increase the mitotic activity of liver cells in rats (Lane & Lieber, 1967; Shulte-Hermann, 1974), to decrease the prothrombin index in rats (Takahashi & Hiraga, 1978a), and to cause haemorrhagic death in rats (Takahashi & Hiraga, 1978b). There are thus many reports on the acute effects of BHT but there have been few studies of its effects after chronic oral administration to animals (Deichmann, Gables, Clemmer, Rakoczy & Bianchine, 1955). Recent studies have indicated that BHT may act as a promoter or an inhibitor in chemical carcinogenesis (Peraino, Fry, Staffeldt & Christopher, 1977; Ulland, Weisburger, Yamamoto & Weisberger, 1973). Thus, the effects of BHT on experimental animals are still controversial. On this basis it seemed important to determine whether BHT has a carcinogenic effects in rats. The present paper describes studies on the chronic toxicity, including the carcinogenicity, of BHT in male and female Wistar rats when given at 1% and 0·25% levels in the diet.

EXPERIMENTAL

Animals and diet. Five-wk-old random-bred Wistar rats (100–200 g) of both sexes were obtained from Nihon Rat Co., Saitama, Japan. The animals were housed five to a plastic cage using wood chips for bedding and were given diet and water *ad lib.* in an air-conditioned room at controlled temperature (23 ± 1°C) and humidity (55 ± 2%) with a 12-hr light-dark cycle. BHT (for additive use) obtained from Wako Pure Chemical Industries Ltd., Osaka was incorporated into Charles River basal diet at concentrations of 1% and 0·25% and the mixtures were made into pellets.

Treatment. Groups comprising 57 rats of each sex were maintained on diets containing 0·25% or 1% BHT from the age of 7 wk. A further 36 rats of each sex were given control diet from the same age. Their weights were recorded weekly and their food consumption was measured at regular intervals. After 104 wk on these diets, the animals were anaesthetized with ether and blood samples were collected for blood smears, red and white blood cell counts, and measurements of haemoglobin and haematocrit, and the following blood biochemical analyses: glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase, cholinesterase, γ-glutamyl transferase (γ-GTP), total protein, albumin/globulin, the thymol turbidity test, total cholesterol, triglyceride, β-lipoprotein, blood urea nitrogen, creatinine, uric acid, total bilirubin, sodium, potassium, chloride and inorganic phosphate. The animals were then killed and the liver, spleen and kidneys were weighed. Selected organs and tissues were fixed in buffered formalin and embedded in paraffin, and sections were stained with haematoxylin and eosin for histological examination. Rats that died or became moribund during the experiment were also autopsied and those that survived for more than

69 wk, the time when the first tumour appeared, were included in the effective numbers.

Statistics. The data were subjected to analyses of variance and the differences between the means were tested with Student's *t*-test. Comparison between groups were made where applicable by the chi-square test.

RESULTS

No persistent reduction in mean food intake was seen in rats of either sex given BHT, but it tended to decrease at wk 84 in animals given BHT at the 0·25% level, at wk 62 to 84 in the 1%-level males and at wk 62, 78 and 96 in the 1%-level females. In males at the lower dose level, a significant reduction in body-weight gain was noted up to wk 36, but thereafter it tended to recover (Fig. 1). The mean body weight of males given BHT at the higher dose level was consistently less than that of controls and the difference was significant up to wk 60. The low-dose females showed reduced body-weight gain at wk 12 and 48. A significant reduction was evident among the high-dose females throughout almost all of the experiment. Increases in both absolute and relative liver weights were observed in treated rats of both sexes. The average relative liver weights at wk 104 were 2·5, 4·1 and 3·7 g/100 g body weight in males and 2·8, 2·9 and 3·5 g/100 g body weight in females of the 0, 0·25% and 1% BHT groups respectively. A reduction in absolute and relative spleen weights was evident in treated females; the females had relative spleen weights of 0·34, 0·21 and 0·23 g/100 g body weight in the 0, 0·25 and 1% BHT groups respectively.

A significant increase in mortality among high-dose males was seen after wk 96 (Fig. 2) but the numbers

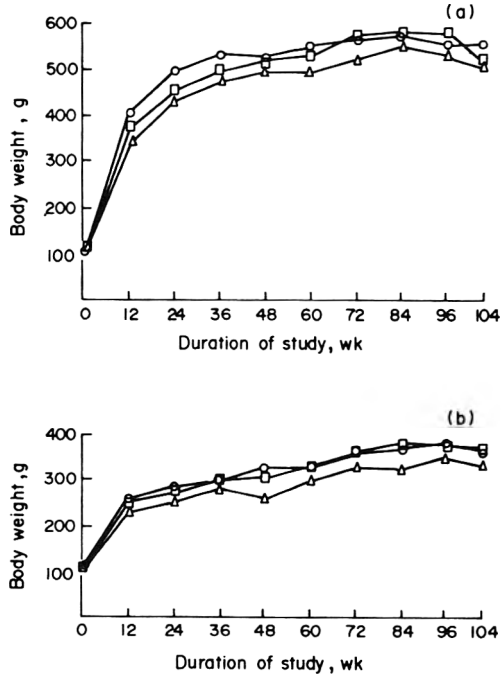

Fig. 1. Mean body weights of (a) male and (b) female rats given control diet (—○—), diet containing 0·25% BHT (—□—) or 1% BHT (—△—).

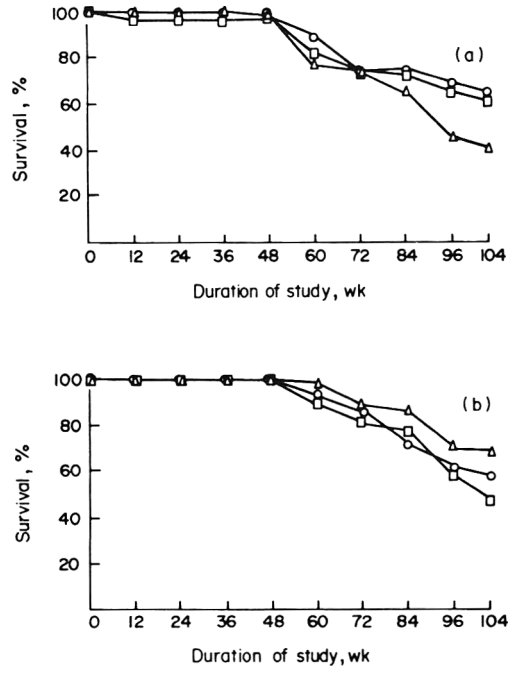

Fig. 2. Survival of (a) male and (b) female rats given control diet (—○—), diet containing 0·25% BHT (—□—) or 1% BHT (—△—).

surviving were sufficient for the haematology and serum biochemistry studies.

At wk 104 the red-blood-cell (RBC) count of females given both levels of BHT was significantly higher ($P < 0.05$) than that in controls (585, 612 and 606 × 10^4/mm^3 at the 0, 0·25 and 1% BHT levels respectively). The white-blood-cell (WBC) count was significantly ($P < 0.05$) lower than that of controls in rats of both sexes at the low-dose level and in female rats at the high-dose level (e.g. in low dose males the count was 18·6 compared with 21·0 × 10^3/mm^3 in the control males). These changes were not dose related.

The serum triglyceride level in treated male rats was significantly lower than that in controls (180, 140 and 137 mg/dl at the 0, 0·25 and 1% BHT levels respectively: $P < 0.01$). Levels were increased compared with those in controls in the case of γ-GTP in male rats (2·8, 3·8, 4·4 mU/dl at the 0, 0·25 and 1% BHT levels respectively: $P < 0.001$) and total cholesterol in female rats (73·4, 99·2, 112 mg/dl at the 0, 0·25 and 1% BHT levels respectively; $P < 0.001$) and total cholesterol in male rats at the 0·25% BHT level (94·8 compared with 83·1 mg/dl in the control rats; $P < 0.05$).

On autopsy, some animals, especially those that died during the experiment, showed various degrees of acute or chronic pneumonia, which was the cause of death in a few animals. The kidneys of both treated and untreated groups of both sexes showed chronic nephropathy: the glomerular basement membrane was thickened, tubules were dilated with colloidal casts, and stromal fibrous tissue had proliferated with slight lymphocytic infiltration. In some females the uterine cavity was dilated with a suppurative or necrotic exudate resulting from inflammatory stenosis of the cervical canal. The testicular gland was atrophic in some male animals. However, since these changes

Table 1. *Tumour incidence in rats fed BHT*

Site/type of tumour	Treatment group... / No. of rats included†...	No. of animals with tumours (%)					
		Males			Females		
		Control 26	0·25% BHT 43	1% BHT 38	Control 32	0·25% BHT 46	1% BHT 51
Liver:	hyperplastic nodule	2 (7·7)	2 (4·7)	1 (2·6)	0	3 (6·5)	3 (5·9)
Pancreas:	carcinoma	0	0	1 (2·6)	0	1 (2·2)‡	4 (7·8)
	islet-cell adenoma	0	1 (2·3)	2 (5·3)	0	0	0
Mammary gland:							
	fibro-adenoma	—	—	—	6 (18·8)	8 (17·4)	8 (15·7)
	adenoma	—	—	—	1 (3·4)	1 (2·2)	1 (2·0)
Uterus:	leiomyoma	—	—	—	1 (3·4)	1 (2·2)	0
	carcinoma	—	—	—	1 (3·1)§	2 (4·3)‖	1 (2·0)‖
Pituitary gland:							
	adenoma	2 (7·7)	3 (7·0)	1 (2·6)	0	6 (13·0)*	3 (11·8)
	carcinoma	0	2 (4·7)	5 (13·2)	3 (9·4)	3 (6·5)	7 (13·7)
Adrenal gland:							
	adenoma	1 (3·8)	3 (7·0)	0	0	2 (4·3)	1 (2·0)
	carcinoma	0	0	0	0	0	1 (2·0)
Others¶		2 (7·7)	2 (4·7)	4 (10·5)	2 (6·3)	4 (8·7)	3 (11·8)
Total		6 (23·1)	13 (30·2)	10 (26·3)	11 (34·4)	25 (54·3)	25 (49·0)

†Animals that survived more than 69 wk were included.
‡Adeno-acanthoma.
§Squamous-cell carcinoma.
‖Adenocarcinomas.
¶The other tumours were a malignant lymphoma of the lung and an osteosarcoma in the limb of one male control; a subcutaneous fibroma and a thyroid adenoma in male rats given 0·25% BHT diet; a subcutaneous fibroma, a subcutaneous lipoma, a chronic myelogenous leukemia and a thyroid adenoma in male rats fed 1% BHT diet; and osteosarcoma in the limb and a thyroid adenoma in a female control; a kidney liposarcoma, a subcutaneous rhabdomyosarcoma, a uterine lipoma and a subcutaneous squamous-cell carcinoma in female rats fed 0·25% BHT diet; and a rhabdomyosarcoma in the retroperitoneum, an osteosarcoma in a limb and a subcutaneous fibroma in female rats fed 1% BHT diet.
The value marked with an asterisk differs significantly (chi-square test) from the corresponding control value ($P < 0·05$).

were found in both BHT-treated and control groups, they were probably not caused by BHT.

The incidence of tumours in male and female rats is summarized in Table 1. The incidence of tumours was slightly, but not significantly, higher in BHT treated groups than in controls. The incidence of hyperplastic nodules and of pancreatic carcinomas in female rats, and of pituitary adenomas or adenocarcinomas in both males and females treated with BHT were higher than those in controls. The increase in the incidence of pituitary adenomas in females given 0·25% BHT diet was significant ($P < 0·05$), but the incidence of pituitary adenomas was not dose-related, and the total incidence of pituitary tumours was not significantly different from that in controls. Thus these tumours do not seem to have been induced by BHT-treatment.

DISCUSSION

In this work some differences were found between the body weights, organ weights and haematology and blood biochemical values of groups fed BHT in the diet and those of animals fed control diets. Administration of BHT has previously been reported to result in decreased body weight and relative spleen weight and increased relative liver and kidney weights in rats without any histopathological changes of the liver or kidney detectable by routine staining (Deichmann *et al.* 1955; Takahashi & Hiraga, 1978c). There are also reports that BHT induced proliferation of the agranular endoplasmic reticulum, perinucleolar heterochromatin aggregation, and enhanced mitotic activity (Lane & Lieber, 1967) and the activities of thymidine kinase and some other enzymes in the liver (Gaunt, Gilbert & Martin, 1965; Saccone & Pariza, 1978). In the present work, we observed significant reduction in the value of triglyceride in male rats, and increases in the levels of γ-GTP in male rats and in total cholesterol in rats of both sexes treated with BHT, but we detected no significant morphological changes in the liver attributable to BHT. Intraperitoneal injection of BHT into mice is reported to produce lung damage followed by proliferation of alveolar cells (Adamson *et al.* 1977; Marino & Mitchell, 1972; Omaye, Reddy & Cross, 1977; Witschi, Kacew, Tsang & Williamson, 1976; Witschi & Sehab, 1974) but in our experiment, we found no tumorous lesions and only a few hyperplastic foci in the alveolar epithelium (in one male and two female rats fed 1% BHT diet). Although the RBC count in female rats was significantly elevated and the WBC count was decreased in rats of both sexes no significant morphological changes were detected in the haematopoietic system.

Tumours were present in some animals given BHT, but their incidence was similar to that in the controls. Consequently, it is concluded that BHT at levels of

0·25% and 1% in the diet, has no carcinogenic effect in rats. These data are consistent with the report of Deichmann *et al.* (1955) that ingestion of diet containing 0·2, 0·5, 0·8 or 1·0% BHT for 24 months had no significant pathological effects on albino rats of either sex. On the other hand, BHT enhanced the induction of liver tumours when given to rats after 2-acetylaminofluorene administration (Peraino *et al.* 1977) and it enhanced the induction of lung tumours when given after a single ip injection of urethan to mice (Witschi *et al.* 1977). However, it inhibited liver-tumour formation when fed with 2-acetylaminofluorene to rats (Ulland, Weisburger, Yamamoto & Weisburger, 1973); it inhibited the development of tumours of the forestomach in mice and of the breast in rats treated with DMBA (Wattenberg, 1972); and it inhibited colon and lung carcinogenesis by 1,2-dimethylhydrazine in mice (Clapp, Bowles, Satterfield & Klima, 1979). In addition, it showed no promoting activity in two-stage mouse-skin tumorigenesis (Berry, DiGiovanni, Juchau, Bracken, Gleason & Slaga, 1978) and has neither enhancing nor inhibitory effect on dimethylhydrazine-induced colon carcinogenesis in rats (Barbolt & Abraham, 1979). It did not affect the development of lungs tumours when given concomitantly with diethylnitrosamine to mice (Clapp, Tyndall, Satterfield, Klima & Bowles, 1978) and had no effect on induction of liver or oesophageal tumours when given with diethylnitrosamine to rats (Ulland *et al.* 1973). As these data show, BHT seems to promote carcinogenesis in the lung and liver, in which it stimulates cell proliferation, but to inhibit carcinogenesis when ingested with a carcinogen. The hepatic microsomal inducers phenobarbital and polychlorinated biphenyls have been shown both to promote and to inhibit hepatocarcinogenesis (Peraino, Fry & Staffeldt, 1971; Makiura, Aoe, Sugihara, Hirao, Arai & Ito, 1974; Nishizumi, 1976). The promoting and inhibiting actions of BHT may similarly result from its induction of hepatic microsomal enzymes (Gilbert & Golberg, 1965; Gilbert, Martin, Gangolli, Abraham & Goldberg, 1969). No clear evidence was obtained that BHT stimulated liver microsomal enzyme activity when ingested by humans at a dose of 140 mg a day for 14 days (Sharratt, Gangolli & Grasso, 1970). However, the amount ingested by rats on the 0.25% BHT diet is equivalent to about 200 times the maximum acceptable daily intake for man set by the WHO (Joint FAO/WHO Expert Committee on Food Additives, 1974). Thus, it seems probable that at high doses BHT may act as a promoter or inhibitor in animals, but that at the maximum acceptable daily intake in man it is neither toxic nor carcinogenic, and does not promote or inhibit carcinogenesis.

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DEVELOPMENTAL NEUROBEHAVIOURAL TOXICITY OF BUTYLATED HYDROXYTOLUENE IN RATS

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Abstract—Butylated hydroxytoluene (BHT) was fed to rats throughout development (from before conception through to day 90 of postnatal life) at levels of 0, 0·125, 0·25 or 0·5% (w/w) in the diet. A similarly treated positive control group was injected on day 12 of gestation with 550 mg/kg of the antimitotic/embryotoxic drug hydroxyurea for reference. Offspring from all groups were reared by their natural dams and were evaluated in a battery of behavioural tests from day 3 to day 90 after birth. BHT at 0·5% in the diet reduced the body weights of dams and of offspring during early development and increased offspring mortality (to 39%) up to 30 days of age. This dose delayed eyelid opening, surface-righting development and limb co-ordination in swimming in males, and reduced female open-field ambulation; however, no significant effects were found after weaning. The lower doses of BHT produced some irregularities in maternal weight (0·25% an increase and 0·125% a decrease) but had no effect on the body weights of offspring. BHT at 0·25% of the diet increased pre- and periweaning mortality (23%), but neither this dose nor the 0·125% dose had any effect on physical or behavioural development or on post-weaning behavioural performance. The positive control group treated with hydroxyurea showed reduced growth prior to weaning, reduced adult brain weight and a slight but nonsignificant increase in pre- and periweaning mortality (10%). This group also exhibited delayed eyelid opening, delayed forward locomotor development and limb co-ordination during swimming, but showed no effects on postweaning behavioural performance. The BHT findings are consistent with the existing toxicological literature that BHT is toxic to growing rodents at doses of 0·25 or 0·5% of the diet with marginal effects at 0·125% of the diet. The behavioural data expand the picture of BHT's toxicity, but do not suggest any disproportionate or special toxicity of BHT for the central nervous system.

INTRODUCTION

A growing awareness of the possible consequences of *in utero* exposure to drugs and other environmental agents on the functional development of the organism has led to the inclusion of tests for behavioural teratogenesis in the reproductive guidelines of Japan, Britain and France. At present these guidelines are quite general, in part because the methodology in this field is not sufficiently well-developed to allow the selection of specific tests of established accuracy. In the United States similar concerns over issues of methodology have prompted the National Toxicology Advisory Committee's Reproduction Panel to state that "At present...Standardization and validation for reliability, sensitivity, and applicability of tests for neurobehavioural toxicity appear to be only beginning, and the imposition of a specific battery of tests may be premature" (Collins, 1978). Nevertheless they advise that "Because some drugs may produce effects not detectable in early life, some of the offspring should be raised to adulthood and tested by neurobehavioural tests" (Collins, 1978). It is clear, therefore, that although the Japanese, European and US recommendations on behavioural assessment are general, and in some instances not even mandatory, there is mounting pressure for their inclusion in the main body of testing requirements. The problem confronting those responsible for testing is that these general recommendations must ultimately be translated into specific tasks.

In 1975 we undertook an FDA-sponsored project to develop and evaluate a battery of neurobehavioural tests applicable to use with food additives. Concurrent validation was provided for by the inclusion of a positive control treatment. We have previously reported our initial experience with this test system using the food additives monosodium glutamate (MSG), calcium carrageenan and aspartame and the positive control treatment hydroxyurea (Vorhees, Butcher, Brunner & Sobotka, 1979a; Brunner, Vorhees, Kinney & Butcher, 1979). Here we report on progress in the use of this test battery to examine the neurobehavioural toxicity of butylated hydroxytoluene (BHT), a widely used antioxidant food additive.

EXPERIMENTAL

Sprague–Dawley rats (Laboratory Supply Co., Indianapolis, IN) were exposed to food additives mixed in powdered Purina rat chow. BHT was supplied by Shell Chemical Co., Houston, TX. Male (200–220 g) and female (200–220 g) rats were acclimatized to the

laboratory for 5 days before assignment to treatment groups whose diets were supplemented with 0·50% BHT, 0·25% BHT, 0·125% BHT or were unsupplemented in the case of the two control groups. Positive control dams were given a single ip injection of 550 mg/kg of hydroxyurea (HU) on day 12 of gestation.

Purina rat chow contains no BHT, but does contain 0·005% of the related antioxidant butylated hydroxyanisole (BHA). The supplemented diets were used continuously after the acclimatization period and both males and females were exposed for not less than 14 days before mating, 1–14 days during breeding, the period of gestation (22 days), and the period of lactation (21 days). After weaning dietary exposure of the offspring to BHT continued throughout the remainder of the experiment (up to day 90 for most animals and somewhat longer for those involved in tests such as passive avoidance).

One breeding pair for each group was enrolled in the study each week until a total of 19 litters were available per group for study. An exception to this occurred in the positive control group due to the high incidence of resorptions produced by HU treatment. Litters of fewer than eight live pups were discarded. Litters of more than 12 were reduced to 12 randomly apart from a sex restriction which tended to equalize the number of males and females.

The rats were weighed at weekly intervals except during breeding, and food consumption was measured every third day in selected rats. The date of birth of all litters was noted and the length of gestation recorded. On the day after birth (day 1) all litters were examined and data collected on litter size, sex distribution, weight and number of dead or malformed offspring. At this time two males and two females were designated and marked for preweaning testing. Two males and two females that were not tested before weaning were designated C and D. Before the fur had grown animals were marked on their backs with a spot of indelible black ink, after the fur developed markings were made with picric acid.

Preweaning observations. Bilateral pinna detachment and upper and lower incisor eruption were measured from day 1. Surface righting reflex was measured daily beginning at day 3 with two trials (30 sec max) per day. Latency between supine and prone positions was measured using a criterion of $\leqslant 2$ sec (Vorhees, Brunner & Butcher, 1979b).

Cliff avoidance was measured daily beginning at day 3. Rats were placed with forepaws over the edge of an elevated horizontal surface. Retraction from the edge begun within 10 sec and completed within 30 sec comprised the criterion (Vorhees *et al.* 1979a). Forward locomotion was observed daily from day 3 until pups could walk with all four legs coordinated (Vorhees *et al.* 1979a). Pivoting locomotion was observed at day 7 during a 60 sec period in which time spent pivoting was recorded (Vorhees *et al.* 1979a).

Auditory startle was elicited by a stimulus of 0·3 sec duration produced by an automobile horn. The rat was placed on a stabilimeter platform 16 cm from the horn inside a sound-insulated chamber. Presence of the startle response was measured by automatic recording of the deviation of the platform and confirmed by the experimenter through an observation window (Vorhees *et al.* 1979b).

Swimming development was assessed on alternate days from day 6 to day 20 (Vorhees *et al.* 1979a). Rats were placed in a tank of water (80°F) for 5 to 15 sec. Measurements were made of direction, angle and limb usage. Direction scores were: sinking (0), floating (1), circling (2), and swimming straight (3). Angle scores were: head submerged (0), nose at surface (1), nose and tip of head above surface (2), ears half-way above surface (3), ears completely above surface (4). Limb usage scores consisted of no paddling (0), paddling with front and rear limbs (1), and paddling with rear limbs only, forelimbs being held stationary (2) (Brunner *et al.* 1979).

Open-field exploration was observed on days 15, 16 and 17 in a circular arena (D = 45·7 cm) for 3-min periods. The acrylic floor surface was marked off into 20 sections in three concentric rings and a central circle. Animals were placed in the inner circle and time taken to leave this area was measured. Section entries were also recorded for the inner, middle and outer rings. The test room was darkened (Vorhees *et al.* 1979b).

Post-weaning behavioural testing. Open-field exploration observations identical to those in the preweaning open-field test but in a much larger arena (D = 91·4 cm) were made on 3 consecutive days, for 3 min/day between days 40 and 45. The floor was clear acrylic and divided on a black surface beneath the acrylic into three concentric rings and a central circle. The rings in turn were divided into sections of equal size by radial lines (Vorhees *et al.* 1979b).

Running wheel activity level was measured in Wahmann galvanized steel cages and the number of revolutions per day was recorded between days 30 and 51 (Vorhees *et al.* 1979a).

Rotorod balancing tests were conducted between days 60 and 65. A plastic rod (D = 11·4 cm) which had a sand surface was linked to a variable speed motor and controller. Shaping was done at low rotational speeds which were gradually increased. The performance criterion was defined as 3 min continuous performance at a setting of 30 rpm. On a trial, if a rat did not remain on the rod at criterion speed after ten attempts, testing was discontinued on that day. Rats were tested for four trials over 2 consecutive days (Brunner *et al.* 1979).

Active avoidance tests were carried out in an aluminium chamber (20·3 × 21 × 17·8 cm) in which a wheel (9·5 cm wide × 7·6 cm diameter) constructed of acrylic and aluminium served as the manipulandum (Kinney & Vorhees, 1979; Brunner *et al.* 1979). A 9 sec white noise warning stimulus preceded footshock (0·75 mA) which could be avoided or escaped by a wheel turn response. The acquisition criterion was 18 avoidances in a single 20-trial session. Extinction trials followed completion of acquisition by 24 hr (warning stimulus alone without shock). The extinction criterion was not more than two avoidances in a single 20-trial session. After a 6-day rest period rats were re-trained to make the avoidance response to the original 18 out of 20 criterion.

Passive avoidance testing was done in a two-chamber apparatus which used the rat's natural tendency to move from a lighted area to one that was dark. For 3 days rats were placed in the lighted chamber (40·6 × 20·3 × 19 cm) and allowed entry to

the dark chamber (30·5 × 20·3 × 19 cm). Entry latencies were recorded. On the first day only, an inescapable footshock (1·0 mA) was delivered to the animal upon entry to the dark compartment. Days 2 and 3 represented retention tests. The maximum latency allowed was 180 sec (Brunner *et al.* 1979).

Histology. At weaning (day 21) extra males were killed by cardiac formalin perfusion under ether anaesthesia. The brain was removed, blocked in three parts and stored in formalin. Brain parts were embedded in paraffin and cut in 6 μm sections which were then mounted and stained with haematoxylin and eosin. Cell counts were made in a microscope at a total magnification of 970 ×. The brains of six rats per group were examined. Cell counts were made in ten separate brain sections in each region. Granule cells of the internal granular layer were counted in the pyramis in mediosagittal sections of the cerebellar vermis. Hippocampal pyramidal cells (CA4) were counted in coronal sections at the level of A3·0. Granule cells of the olfactory bulbs were counted in matched horizontal sections (Brunner *et al.* 1979).

At 90 days of age rats representing each group were killed by ether overdose and exsanguinated through the heart. The eyes were removed and weighed. The brain was removed and the cerebellum, brainstem and cerebrum including olfactory bulbs were separately weighed.

Statistical analysis. Analyses of variance were obtained on the majority of data with Duncan's comparisons made in the event of significant F ratios. In order to correct for unequal group sizes, the unweighted means solution was applied to all analyses of variance. Corresponding adjustments of Duncan's test were made using the procedure of Kramer (1956). The minimum acceptable level of significance was $P < 0·05$. On preweaning tests, data on individual subjects was averaged and litter was used as the unit of analysis. On postweaning tests, individual animals were used as the unit of analysis. Frequency data (mortality) were analysed by Fisher's test for uncorrelated proportions (Guilford, 1965).

RESULTS

Physical measurements

Body weights proved to be more sensitive to treatment effects than food consumption, therefore food consumption data is not presented.

There were no significant effects of BHT on body weights during the 2-week prebreeding exposure period. Weights were not recorded during breeding, but as can be seen in Table 1, significant weight differences did appear during gestation. Animals in the 0·50%-BHT group were 4–5% lighter on days 7 and 14 of gestation than the negative controls. In contrast, the 0·25%-BHT group was 3–4·5% heavier than the negative control group on days 7 and 14 of gestation. During lactation the 0·50%-BHT group showed significantly reduced weights averaging from 6·5% less than the controls on the day after parturition to 10% less at weaning. The 0·125%-BHT group was 4·5% lighter than the control group on the twenty-first day of lactation. The 0·25%-BHT and positive control groups showed no significant weight changes during lactation.

The growth of the offspring is depicted in Table 2. During the preweaning period the 0·50%-BHT group showed the largest and most persistent weight reductions. The only other group that showed a significant weight reduction in the offspring was in the positive control group and in this instance the effect was more pronounced in the females than in the males. During the postweaning period only the 0·50%-BHT group showed significantly reduced body weights. This effect gradually diminished, however, from a maximum reduction of 29% at 30 days to 24% at 37 days, 17% at 44 days and finally 10% at 90 days of age among males, the latter difference not being significant. There were no significant weight differences in any group at day 90.

Male brain weights at day 90 are shown in Table 3. There were no significant differences in cerebellar or brainstem weights, but the cerebrum was significantly reduced in the HU group by 15·5%. Trends towards similar reductions in the cerebellum and brainstem

Table 1. *Body weights of rat dams during gestation and lactation*

Parameter		Treatment group...	Body weight (g) Negative control	Positive control	0·125% BHT	0·25% BHT	0·50% BHT
			Gestation				
		No. of rats...	13	17	17	22	22
Gestation day	0		249 ± 3	249 ± 4	249 ± 3	255 ± 4	244 ± 3
	7		275 ± 5	276 ± 5	275 ± 3	285 ± 4*	265 ± 3*
	14		305 ± 5	307 ± 5	309 ± 4	319 ± 6*	289 ± 4*
			Lactation				
		No. of rats...	19	7	19	19	19
Lactation day	1		291 ± 5	293 ± 7	289 ± 4	293 ± 7	272 ± 5**
	7		305 ± 5	299 ± 10	297 ± 4	302 ± 7	281 ± 5**
	14		308 ± 7	305 ± 7	300 ± 5	312 ± 7	283 ± 5**
	21		313 ± 7	321 ± 7	299 ± 6**	305 ± 7	282 ± 4**

Values are means ± SEM and those marked with asterisks differ significantly from the negative control value (*$P < 0·05$; **$P < 0·01$).

Table 2. *Body weights of offspring*

Treatment	No. of litters	Body weight (g)									
		Preweaning period					Postweaning period				
		Age (days)... 1	7	14	21	No. of rats	Age (days)... 30	37	44	No. of rats	90†
Males											
0·50% BHT	19	7·1 ± 0·2	12·6 ± 0·4**	21·3 ± 0·8**	30·0 ± 1·3**	11	52 ± 5**	84 ± 4**	122 ± 4	12	309 ± 11
0·25% BHT	18	7·5 ± 0·2	14·4 ± 0·4	24·7 ± 0·7	35·1 ± 1·8	13	78 ± 3	106 ± 7	146 ± 9	12	330 ± 10
0·125% BHT	19	7·9 ± 0·2	15·3 ± 0·3	25·3 ± 0·5	36·1 ± 1·0	16	74 ± 2	114 ± 3	152 ± 4	13	325 ± 7
Positive control	7	6·1 ± 0·3	12·9 ± 0·6**	24·7 ± 1·5	37·0 ± 2·1	4	79 ± 7	122 ± 9	167 ± 11	7	312 ± 15
Negative control	19	7·5 ± 0·2	15·1 ± 0·4	25·4 ± 0·6	35·8 ± 0·9	10	73 ± 5	111 ± 7	147 ± 9	15	342 ± 5
Females											
0·50% BHT	19	6·8 ± 0·1	11·9 ± 0·4**	20·2 ± 0·7	27·8 ± 1·2**	15	51 ± 4**	82 ± 4*	109 ± 6	—	—
0·25% BHT	18	7·2 ± 0·3	13·8 ± 0·5	23·1 ± 0·8	33·1 ± 1·3	10	63 ± 4	92 ± 6	126 ± 5	—	—
0·125% BHT	19	7·6 ± 0·2	14·5 ± 0·3	24·7 ± 0·4	34·2 ± 1·1	14	66 ± 2	102 ± 4	129 ± 5	—	—
Positive control	7	5·5 ± 0·4	11·9 ± 0·6**	22·8 ± 1·2	28·1 ± 4·8**	4	76 ± 3	113 ± 4	138 ± 4	—	—
Negative control	19	7·1 ± 0·1	14·5 ± 0·3	24·5 ± 0·6	34·9 ± 1·0	11	65 ± 3	98 ± 4	120 ± 6	—	—

†Females were not used for adult brain or body-weight determinations in this experiment.
Values are means ± SEM and those marked with asterisks differ significantly from the negative control value (*$P < 0·05$; **$P < 0·01$).

Table 3. *Brain and eye weights of offspring at 90 days of age*

Treatment	No. of rats	Weight (mg)				
		Cerebellum	Brainstem	Cerebrum	Total brain	Eyes
0·50% BHT	12	277 ± 9	205 ± 7	1404 ± 23	1891 ± 30	126 ± 1
0·25% BHT	12	285 ± 6	214 ± 9	1394 ± 21	1893 ± 30	130 ± 2
0·125% BHT	13	290 ± 7	199 ± 8	1424 ± 31	1913 ± 39	128 ± 2
Positive control	7	274 ± 4	189 ± 8	1228 ± 57**	1691 ± 57**	123 ± 2*
Negative control	15	294 ± 6	214 ± 4	1454 ± 20	1962 ± 23	130 ± 1

Values are means ± SEM and those marked with asterisks differ significantly from the negative control value (*$P < 0.05$; **$P < 0.01$).

combined with the cerebum effect contributed to an overall significant reduction in total brain weight in the HU group (14%). The HU group also had significantly reduced eye weights (5%). None of the BHT groups showed significant changes in either brain or eye weights.

Neuronal cell counts in 21-day-old males showed no differences in cell density in either the cerebellar or hippocampal regions in any group. In the olfactory bulbs there was an isolated significant increase in cell density in the 0·125%-BHT group compared to negative controls of 17·5%.

Mortality figures are shown in Table 4. Essentially all of the deaths occurred during the pre- and peri-weaning period, days 1–30. Both the 0·50%-BHT and 0·25%-BHT groups had substantial mortality rates and the effects were dose dependent. The 0·125%-BHT and positive control groups mortality rates were also elevated, but not significantly. There was also indirect evidence of increased prenatal mortality in the 0·50%-BHT group, in that mean litter size at birth in this treatment group was 10·0 ± 0·3 compared with negative controls which averaged 12·0 ± 0·4 ($P < 0.05$). The positive control group showed a similar reduction averaging 10·3 ± 0·7 offspring per litter ($P < 0.05$). Length of gestation and offspring sex ratio, however, were not significantly affected.

Behavioural measurements and development milestones

As can be seen in Tables 5 and 6 only the 0·50%-BHT and HU groups produced any significant alterations in early development. The 0·50-BHT group showed a significant delay in surface-righting development of nearly a day and a half, a significant delay in eyelid opening of more than a day (Table 5), a significant delay in the development of forelimb inhibition in the maturation of swimming in males on days 18 (Table 6) and 20 (not shown) and a significant reduction in exploration (ambulation) on the first day of open-field testing in females (Table 6). The HU group showed nearly a 2-day delay in the development of co-ordinated forward locomotion, slightly over half a day's delay in eyelid opening (Table 5), and slow onset of swimming with all four legs (Table 6). There were no significant effects on swimming direction or angle development, nor in open-field starting latencies, therefore these data are not shown.

The results of postweaning activity testing are shown in Table 7. There were no significant differences in open-field ambulation, rearing, defaecation or starting latencies and no differences in activity wheel running. The results of the learning tests are shown in Table 8. Females performed significantly better on the rotorod than males, but there were no significant treatment-related effects. There were no significant treatment-related effects on either active or passive avoidance learning, although there was a trend towards diminished 48-hr retention performance in the males of the 0·50%-BHT and 0·25%-BHT groups.

DISCUSSION

The antioxidant BHT has been used as a food additive for over 20 yr. Developmental toxicological studies have demonstrated reduced weight gain, increased liver size and elevated serum cholesterol in

Table 4. *Postnatal mortality*

Treatment	Percentage of progeny (that were alive on day 1) that died from days		
	1 to 30	30 to 90	1 to 90
0·50% BHT	39*	2	41
0·25% BHT	23*	1	24
0·125% BHT	9	0	9
Positive control	10	0	10
Negative control	3	3	6

†The day of birth was day 0.
The values marked with asterisks differ significantly from the negative control value (*$P < 0.01$).

Table 5. *Preweaning development*

Parameter	Treatment group...	Mean day on which all tested members of each litter met the various criteria: Negative control	Positive control	0·125% BHT	0·25% BHT	0·50% BHT
No. of litters		18–19	7	19	18–19	18–19
Pinna detachment		3·1 ± 0·2	3·0 ± 0	2·9 ± 0·1	2·8 ± 0·1	3·4 ± 0·3
Surface righting		8·0 ± 0·2	8·4 ± 0·5	8·0 ± 0·2	8·5 ± 0·3	9·4 ± 0·5*
Cliff avoidance		8·0 ± 0·5	9·3 ± 0·8	9·2 ± 0·1	9·3 ± 0·4	8·1 ± 0·7
Incisor eruption		10·9 ± 0·2	11·0 ± 0	10·4 ± 0·3	10·8 ± 0·3	11·1 ± 0·3
Forward locomotion		9·6 ± 0·2	11·3 ± 0·3*	10·0 ± 0·3	9·7 ± 0·2	10·4 ± 0·4
Auditory startle		12·7 ± 0·3	12·9 ± 0·3	12·3 ± 0·2	12·7 ± 0·3	13·2 ± 0·3
Eyelid opening		15·3 ± 0·2	15·9 ± 0·4*	15·7 ± 0·2	15·4 ± 0·3	16·5 ± 0·3**
Pivoting: time (sec)		11·0 ± 1·9	15·7 ± 3·5	16·5 ± 2·1	16·2 ± 2·2	10·9 ± 1·8
no. of 90° turns		3·8 ± 0·6	3·7 ± 1·3	4·0 ± 0·7	3·9 ± 0·6	2·6 ± 0·3

†Four pups from each litter were tested.
Values are means ± SEM and those marked with asterisks differed significantly from the negative control value (*$P < 0·05$; **$P < 0·01$).

F_0- and F_1-generation rats fed diets containing up to 0·5% BHT (Brown, Johnson & O'Halloran, 1959; Frawley, Kohn, Kay & Calandra, 1965; Johnson & Hewgill, 1961). We have also found reduced weight-gain, accompanied by increased mortality up to 30 days of age in F_1 generation rats fed 0·25 or 0·5% BHT in the present study. It has also been noted that the effects of BHT on growth are a function of the interaction of BHT dose and dietary lipid content; for example, differential weight reductions were obtained with a constant BHT dose of 0·5% when the lipid content was varied from 28%, which produces a severe weight reduction, to 8%, which produces only a moderate weight reduction (Brown *et al.* 1959). This interaction largely disappears, however, at lower doses of BHT (Brown *et al.* 1959; Frawley *et al.* 1965). Brown *et al.* (1959) reported a low incidence (15%) of anopthalmia from BHT. However, this effect has not been replicated in subsequent studies (Clegg, 1965; Johnson, 1965), including our own in which we found no evidence of ocular malformations. Other reported effects of BHT on development have included reduced litter size, lengthened gestation and reduced fertility in mice (Johnson, 1965), though it has been suggested that these parameters are unaltered in rats (Brown *et al.* 1959; Frawley *et al.* 1965; Johnson & Hewgill, 1961). In the present study we confirmed in rats the finding of reduced litter size, but we found no effect on the length of gestation. We did not measure fertility in this experiment.

Only one previous behavioural toxicology experiment could be found in the literature on BHT and it was done in mice rather than in rats (Stokes & Scudder, 1974). In this study, 0·5% BHT was fed to mice beginning prenatally and extending throughout adult testing of the offspring. The BHT-treated offspring displayed decreased sleeping time, increased fighting incidences and decreased active avoidance acquisition. Associated reductions in brain biogenic amines were also reported (Stokes, Scudder & Karczmar, 1972). Unfortunately, Stokes & Scudder (1974) failed to report the mortality and growth rates of their animals. In the present study we did not measure somnolence or aggression, but we did test active avoidance. In contrast to Stokes & Scudder (1974) we found no reduction in avoidance learning in BHT-fed animals. We did, however, find several effects of BHT that have not been measured before. These include delayed surface righting, delayed eyelid opening, delayed onset of the adult swimming pattern and reduced initial exploration in the open-field. All of these effects occurred during the preweaning period, which was not examined by Stokes & Scudder (1974).

Based on our current and previous data we conclude that BHT has marked developmental toxicity at high doses. Our data suggest, however, that even at high doses BHT has little effect on eventual adult behaviour. It appears that animals that survive the pre- and immediate postweaning period recover from the adverse effects of BHT. This interpretation must be tempered, however, by two considerations. The fact that the high- and mid-dose BHT groups exhibited substantial attrition may have resulted in a biased population of survivors for use in the adult tests in those groups. It remains to be proven whether the adult tests used here are of greater, lesser or of equal sensitivity to those used prior to weaning. It is conceivable that the adult tests are substantially less sensitive and if this were true then the potential effects of BHT on adult behaviour might have been underestimated by the current test system. We now have preliminary evidence that the latter possibility may, in fact, be correct (Butcher, Wootten & Vorhees, 1980).

The battery of tests used here cannot be regarded as final; nevertheless, this battery does meet several of the most important criteria that must be required of any screening system. One crucial aspect is comprehensiveness. The present system contains a wide range of tests and covers all the major areas thus far required in behavioural teratology studies. That is, Japan requires tests of "locomotion, learning, sensory functions and emotionality". The present battery examines various aspects of locomotor behaviour in the tests of forward locomotion development, pivoting, open-field exploration, activity-wheel running and even swimming locomotion. Learning is represented

Table 6. *Pre-weaning activity tests, paddling development in swimming and open-field ambulation*

Treatment	Sex	Swimming—rating for paddling development: No. of litters	Age (days)... 6	10	14	18	Open-field ambulation rating: No. of rats	Age (days)... 15	16	17
0·5% BHT	M	19	0·7 ± 0·1	1·0 ± 0·1	1·0 ± 0·1	1·5 ± 0·1**	19	21·1 ± 3·3	39·3 ± 4·5	45·7 ± 6·0
	F	19	0·8 ± 0·1	1·0 ± 0·0	1·0 ± 0·0	1·7 ± 0·1	18	30·7 ± 5·0*	44·1 ± 5·6	43·2 ± 5·0
0·25% BHT	M	19	0·9 ± 0·1	1·0 ± 0·0	1·0 ± 0·0	1·8 ± 0·1	17	43·7 ± 5·3	46·6 ± 6·0	56·8 ± 6·0
	F	19	0·9 ± 0·1	1·0 ± 0·0	1·1 ± 0·0	1·8 ± 0·1	18	48·6 ± 7·0	55·0 ± 7·3	64·9 ± 6·6
0·125% BHT	M	19	0·9 ± 0·0	1·0 ± 0·0	1·1 ± 0·0	1·8 ± 0·1	18	38·9 ± 5·3	52·4 ± 6·0	51·6 ± 6·2
	F	19	0·8 ± 0·1	1·0 ± 0·0	1·1 ± 0·0	1·8 ± 0·1	18	38·3 ± 4·5	42·8 ± 5·4	48·4 ± 6·2
Positive control	M	7	0·6 ± 0·2*	0·7 ± 0·0**	1·1 ± 0·1	1·9 ± 0·1	7	38·3 ± 8·2	42·7 ± 8·0	61·9 ± 8·1
	F	6	9·9 ± 0·1	1·0 ± 0·0	1·2 ± 0·1	1·9 ± 0·1	6	50·8 ± 8·3	65·0 ± 9·1	69·8 ± 7·8
Negative control	M	19	0·8 ± 0·1	1·0 ± 0·0	1·1 ± 0·0	1·8 ± 0·1	18	39·5 ± 5·3	42·2 ± 6·2	47·9 ± 5·9
	F	19	0·9 ± 0·1	1·0 ± 0·0	1·0 ± 0·0	1·7 ± 0·1	18	46·1 ± 5·2	57·6 ± 5·7	55·6 ± 5·9

Values are means ± SEM and those marked with asterisks differ significantly from the negative control value (*$P < 0.05$; **$P < 0.01$). SEM values of less than 0·05 are shown as 0·0.

Table 7. *Post-weaning activity tests*

Treatment	Sex	Open field activity scores*					Activity wheel scores†	
		No. of rats	Ambulation	Rearing	Defaecation	Starting latencies	No. of rats	Revolutions
0·50% BHT	M	11	50·1 ± 6·8	11·7 ± 2·2	0·8 ± 0·5	3·0 ± 2·1	11	850 ± 68
	F	12	57·8 ± 5·1	15·9 ± 2·7	1·1 ± 0·4	1·4 ± 0·4	11	1666 ± 404
0·25% BHT	M	13	57·4 ± 4·0	16·3 ± 2·6	1·6 ± 0·5	1·2 ± 0·3	15	1068 ± 228
	F	11	59·4 ± 3·0	15·8 ± 2·1	1·4 ± 0·5	1·4 ± 0·3	15	2076 ± 467
0·125% BHT	M	18	59·4 ± 3·9	14·5 ± 2·1	1·3 ± 0·04	1·1 ± 0·2	17	1067 ± 160
	F	16	60·9 ± 3·7	15·3 ± 2·0	1·4 ± 0·4	1·5 ± 0·5	16	1628 ± 336
Positive control	M	6	51·1 ± 8·0	14·1 ± 2·9	0·9 ± 0·5	1·8 ± 0·8	7	767 ± 105
	F	7	59·6 ± 6·3	14·6 ± 3·1	1·7 ± 0·6	2·2 ± 1·0	6	2289 ± 754
Negative control	M	16	46·9 ± 4·9	12·3 ± 1·9	1·1 ± 0·4	1·4 ± 0·2	17	1384 ± 402
	F	15	60·4 ± 4·3	15·5 ± 1·8	1·2 ± 0·4	1·3 ± 0·2	17	2075 ± 339

*The scores for 3 days of testing are combined.
†The scores for 20 days of testing are combined.

in tests of active and passive avoidance behaviour and even to some extent in rotorod performance. Sensory functions are represented in startle and active avoidance for auditory cues, rotorod and swimming for equilibrium, and passive avoidance for vision. Vision, however, is probably not adequately represented since passive avoidance requires only the most gross kind of light-dark visual perception, nevertheless, it appears to be superior to a measure such as pupillary reflex. We are actively working on a new test for vision. Finally, emotionality is represented in the two open-field tests by the interaction of activity and defaecation measures which constitute the classic description of emotionality in rats (Whimby & Denenberg, 1967). Britain and France also require behavioural testing and have identical behavioural specifications. Each requires tests of "auditory, visual and behavioural impairment". Audition and vision are represented in the current system as described above and behavioural impairment may include any of the tests in the present battery for activity, balance or learning, though the term 'impairment' was probably intended to imply the notion of a 'learning impairment'.

A second consideration for an adequate test battery is practicability. The present system is time-consuming and labour intensive. Only the active avoidance test is fully automated, most of the tests in our battery being completely or at least partially manual. At present, however, there is really no satisfactory way around this problem in behavioural testing. More research is needed in this area in order to streamline and automate behavioural tests of proven utility, meanwhile there is no substitute for skilled judgement.

A good test battery must also show sensitivity which is a function of the reliability and the validity of the test. First, let us consider reliability. Reliability involves within-study reliability, (i.e. the minimum variability that results from inherent individual differences), and reproducibility or the constancy of measurement devices at different times both within a laboratory and between laboratories. Activity wheels are a good example of a test in the current battery with high inherent variability. As seen in Table 7, the standard errors of some groups were almost one third of the size of the mean. This raises serious questions in our view about the ultimate utility of activity wheels for toxicological experiments. Intralaboratory reliability appears to be fairly good if one compares our current negative controls to those in our first experiment with this battery (Vorhees *et al.* 1979a). For example, the scores of the present negative controls versus the previous ones for pinna detachment are 3·1 ± 0·2 vs. 3·0 ± 0·2, for incisor eruption 10·9 ± 0·2 vs. 10·8 ± 0·2, for eyelid opening 15·3 ± 0·2 vs. 15·4 ± 0·2 and for startle 12·7 ± 0·3 vs. 12·3 ± 0·2. Comparability was also good for swimming development as well as on several adult tests, including active avoidance, passive avoidance, and post-weaning open-field exploration. Tests that did not replicate well included cliff avoidance (8·0 ± 0·5 vs. 6·8 ± 0·3), righting (8·0 ± 0·2 vs. 9·0 ± 0·4), forward locomotion (9·6 ± 0·2 vs. 7·9 ± 0·4) and of the adult tests, the rotorod and the activity wheel. The remaining tests could not be compared, preweaning open-field exploration was not included in the previous study, pivoting was redefined in the present study and visual placing was not included in the present study. It is too early in the development of the current test battery to determine why the intralaboratory reliability of some tests fluctuated, because there were many potential contributing factors: changes in technical personnel, changes in physical facilities and changes in test procedures (although we tried to minimize the latter). The subject of interlaboratory reliability is beyond the scope of this project although it is noteworthy that research in this crucial area is a rarity in the behavioural sciences, though some promising beginnings have been made (Butcher, Hoar, Nolan & Vorhees, 1979).

The validity of the test battery is ultimately the most fundamental issue and it is also the most difficult to establish. It is one of the anomalies of science that the ability to measure things sometimes precedes our understanding of what we have measured, and this seems to be true in much of psychoteratology. Nevertheless, the most widely accepted approach to the validity problem in psychoteratology thus far has been to test concurrently one or more compounds with documented toxic effects along with the com-

Table 8. *Post-weaning behavioural tests of rotorod balance and active and passive avoidance learning*

		Rotorod*		Active avoidance†				Passive avoidance‡			
Treatment	Sex	No. of animals	Time (sec)	No. of animals	Acquisition	Extinction	Re-acquisition	No. of animals	Training	24-hr retention	48-hr retention
0·50% BHT	M	13	49·4 ± 12·3	15	3·1 ± 0·5	3·9 ± 0·8	1·6 ± 0·2	8	20·7 ± 4·4	90·8 ± 26·6	72·0 ± 24·5
	F	13	93·6 ± 14·4	6	2·0 ± 0·3	5·2 ± 1·6	1·4 ± 0·2	7	19·5 ± 4·6	101·6 ± 29·0	83·2 ± 28·6
0·25% BHT	M	17	54·1 ± 12·5	12	2·4 ± 0·5	4·1 ± 1·3	2·0 ± 0·3	12	17·9 ± 4·6	76·8 ± 20·8	54·7 ± 17·7
	F	17	103·0 ± 15·6	8	2·8 ± 0·6	4·7 ± 0·9	1·7 ± 0·3	10	17·0 ± 5·4	100·0 ± 27·0	86·1 ± 25·8
0·125% BHT	M	20	65·4 ± 14·5	17	2·9 ± 0·4	2·2 ± 0·5	2·1 ± 0·5	15	13·5 ± 2·8	75·4 ± 17·7	89·1 ± 18·8
	F	20	93·1 ± 16·0	12	2·2 ± 0·3	6·2 ± 3·1	1·2 ± 0·2	14	15·9 ± 3·0	80·7 ± 13·1	58·5 ± 11·9
Positive control	M	7	38·6 ± 15·1	6	3·0 ± 1·0	1·5 ± 0·3	2·0 ± 0·5	4	19·3 ± 8·1	138·6 ± 44·4	97·8 ± 47·8
	F	6	92·3 ± 16·5	—	—	—	—	4	8·3 ± 2·0	76·3 ± 35·4	47·5 ± 14·5
Negative control	M	17	62·8 ± 15·7	13	2·9 ± 0·5	2·9 ± 0·5	1·5 ± 0·2	14	21·0 ± 3·7	119·5 ± 19·0	117·9 ± 19·4
	F	20	70·6 ± 12·3	11	3·1 ± 0·8	4·6 ± 2·7	1·2 ± 0·2	14	18·7 ± 4·4	70·7 ± 16·9	64·2 ± 17·6

*Average time on rotating rod for all four trials.
†No. of days taken to reach criterion level of performance in each phase of testing.
‡Time taken for entry into the dark compartment (sec).
Values are means ± SEM.

pounds of unknown toxicity (Butcher, 1976). The reference compound used in the present study was HU which was a mixed success. The HU group showed delayed forward locomotion, delayed eyelid opening and delayed early swimming development, but showed no effects in any of the adult tests. While it is not reasonable to expect any single positive control treatment to affect all the dependent measures used, nonetheless, HU does not seem to be an ideal positive control treatment based on this and our previous experiment using an identical procedure (Vorhees *et al.* 1979a). It is clear from this experience that progress in the area of method validation in neurobehavioural toxicology will, to a considerable extent, depend upon the development of a set of good positive control treatments, a process that is receiving far too little attention at the present time.

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EFFECT OF DIETARY TIN ON ZINC, COPPER AND IRON UTILIZATION BY RATS

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Abstract—Male, weanling Sprague–Dawley rats were fed a diet containing 206 μg tin (as stannous chloride)/g feed for 21 days. The rats fed the test diet lost significantly more zinc in their faeces and retained significantly lower levels of zinc in their tibias and kidneys than rats fed the control diet (which contained 1 μg tin/g). The rats fed the tin-supplemented diet retained significantly higher levels of tin in their kidneys and tibias and significantly lower levels of copper in their kidneys than the control animals.

INTRODUCTION

Large doses of tin, especially of alkyl tin derivatives, are toxic to man and animals (Barnes & Stoner, 1959; Monier-Williams, 1949; Schroeder, Balassa & Tipton, 1964). Occasionally, there are reports of individuals who consumed high levels of tin in food and who developed toxic symptoms, usually gastro-intestinal distress (Benoy, Hooper & Schneider, 1971; Warburton, Udler, Ewert & Haynes, 1962). Both Barnes & Stoner (1959) and Monier-Williams (1949) stated that tin in food was "not a potential health problem" if the tin concentration in the food was less than 250 μg/g. However, they generally considered as health problems only acute and severe signs or symptoms such as death, paralysis and gastro-intestinal disturbances.

More recently several groups of investigators have demonstrated subacute symptoms resulting from chronic oral exposure to tin. de Groot (1973), de Groot, Feron & Til (1973) and Fritsch, de Saint Blanquat & Derache (1977a) observed reduced growth, food intake, haematocrits and haemoglobin levels in rats fed high levels of certain tin salts (3000–6000 μg/g feed) for 1 month. de Groot (1973) also demonstrated low haemoglobin levels, serum iron levels and growth rates in rats fed diets containing 150 μg inorganic tin/g feed for 1 month. The haematological changes that occurred when rats were fed high levels of tin could be diminished or prevented by enriching the diets with iron and/or copper. However, iron and copper supplements did not prevent the growth depression induced by feeding elevated levels of tin (de Groot, 1973; de Groot *et al.* 1973).

The purpose of this study was to determine whether the addition of tin to the diet of rats depressed their apparent absorption and retention of zinc, as well as those of copper and of iron. We considered that tin might impair zinc absorption because a number of dietary factors that affect copper and iron absorption also affect zinc absorption; also growth depression and inanition are common signs of zinc deficiency (Underwood, 1977).

EXPERIMENTAL

The control diet contained 50·0% sucrose, 20·0% lactalbumin (Teklad Test Diets, Madison, WI), 15·3% cornstarch, 5·0% non-nutritive fibre-cellulose (Teklad Test Diets), 5·0% corn oil (Teklad Test Diets), 3·5% Williams-Briggs modified mineral mixture (Teklad Test Diets), 1·0% AIN-76 vitamin mixture (American Institute of Nutrition, 1977), and 0·2% choline dihydrogen citrate. Stannous chloride (Mallinckrodt, Inc., Paris, KY) was added to the diet that was fed to the test group. By analysis, the control and test diets were found to contain 1 and 206 μg tin/g, respectively. The trace element content of the diets was found by analysis to be 36·8 μg iron/g, 23·5 μg zinc/g and 6·2 μg copper/g.

Male Sprague–Dawley weanling rats (Teklad Test Diets) were fed the control diet for 4 days before the start of the study. The rats were then randomly assigned to the test group (n = 7) or the control group (n = 8). The animals were housed individually in stainless-steel wire-bottomed metabolism cages. Deionized water was given *ad lib.* The rats were fed *ad lib.* but their food intake was measured daily. They were weighed twice a week. Faeces were collected during days 7–10 and days 14–17. On day 22 the rats were killed by decapitation. The tibias, kidneys, and livers were removed, cleansed of all adhering matter, weighed and frozen.

Tissues and five samples of each diet were dry ashed and analysed by atomic-absorption spectrophotometry for their zinc, copper and iron contents (West, Nonnamaker & Greger, 1979). Liver samples obtained from the National Bureau of Standards (NBS) were dry ashed and processed in the same manner. The mean (n = 4) analysed zinc, copper, and iron contents of liver samples were 101, 104, and 101%, respectively, of the NBS certified values.

The ashed kidney samples were also analysed for calcium by atomic-absorption spectrophotometry. Ashed samples and standards were diluted so as to contain 0·1% lanthanum (Perkin-Elmer Corp., 1976).

The faecal samples, right tibias and right kidneys

Table 1. *Effect of dietary tin on zinc retention by rats*

Parameter		Mean values† for rats fed: Tin-supplemented diet	Control diet
Tibia zinc:	concentration (μg/g)	162 ± 8**	177 ± 10
	content (μg/tibia)	54·2 ± 4·1***	62·0 ± 3·2
Kidney zinc:	concentration (μg/g)	21·7 ± 2·7*	25·6 ± 3·8
	content (μg/kidney)	17·8 ± 3·2	21·4 ± 3·7
Liver zinc:	concentration (μg/g)	26·5 ± 2·3	24·9 ± 2·7
	content (μg/liver)	251 ± 16	267 ± 36
Faecal zinc:	days 7–10 (μg/day)	188 ± 31*	154 ± 19
	days 14–17 (μg/day)	251 ± 20*	216 ± 29

†Values are means ± 1 SD for groups of seven test and eight control rats. The control and test diets contained 1 and 206 μg tin/g, respectively. Test-group values marked with asterisks differ significantly (Student's *t*-test) from the corresponding values for the controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

were wet ashed with nitric acid, perchloric acid, sulphuric acid and hydrogen peroxide (Oh, Ganther & Hoekstra, 1974). The zinc, copper and iron contents of the faecal samples were determined by atomic-absorption spectrophotometry. The tin contents of all of these samples were determined by a colorimetric procedure that used pyrocatechol violet (Corbin, 1970 & 1973). NBS does not certify the tin content of the liver samples, so recoveries were conducted. The mean recovery of tin added to six tissue samples was 102%.

Means, standard deviations, and Student's *t*-tests were calculated (Steel & Torrie, 1960).

RESULTS

The addition of tin to the diet did not significantly affect food consumption or weight gain. The rats fed the tin-supplemented diet consumed 12·8 ± 0·9 g feed/day (mean ± 1 SD) and gained 6·0 ± 0·4 g/day, whereas the rats fed the control diet consumed 12·9 ± 0·9 g/day and gained 6·2 ± 0·4 g/day.

The addition of tin to the diet did affect the retention of zinc (Table 1). The concentration and content of zinc in the tibias and the concentration of zinc in the kidneys of the rats fed the test diet were significantly less than those of the rats fed the control diets. The addition of tin to the diet did not affect the level of zinc retained in the livers of rats. The rats fed the test diet lost significantly more zinc in their faeces during both collection periods than the control rats.

The addition of tin to the diet also affected the retention of copper (Table 2). The copper concentration and content of the kidneys and the copper content of the livers of the animals fed the tin-supplemented diet were significantly lower than those of the control rats. The dietary treatments did not affect significantly the concentration of copper in the livers of these animals.

The animals fed the test diet tended to lose more copper in their faeces than the control rats, especially durings days 14–17. However, the differences were not statistically significant.

The dietary treatments had no effect on the retention of iron in the kidneys or on faecal losses of iron during either collection period (Table 3). However, the concentration of iron in the livers of the treated rats was significantly greater than those in the livers of control rats.

The levels of tin in the tibias and kidneys of rats fed the tin-supplemented diets were much greater than the levels of tin in the tissues of control rats (Table 4). However, the calcium concentration in the kidneys of the rats fed the tin-supplemented diet (58·8 ± 8·1 μg/g, mean ± 1 SD) was similar to that of the control rats (59·0 ± 6·7 μg/g).

Table 2. *Effect of dietary tin on copper retention by rats*

Parameter		Mean values† for rats fed: Tin-supplemented diet	Control diet
Kidney copper:	concentration (μg/g)	5·7 ± 0·9**	8·3 ± 1·8
	content (μg/kidney)	4·6 ± 1·0**	6·9 ± 1·6
Liver copper:	concentration (μg/g)	3·1 ± 0·5	3·2 ± 0·3
	content (μg/liver)	29·4 ± 3·1*	34·8 ± 5·1
Faecal copper:	days 7–10 (μg/day)	66 ± 7	64 ± 5
	days 14–17 (μg/day)	91 ± 12	80 ± 8

†Values are means ± 1 SD for groups of seven test and eight control rats. The control and test diets contained 1 and 206 μg tin/g, respectively. Test-group values marked with asterisks differ significantly (Student's *t*-test) from the corresponding values for the controls (*$P < 0.05$; **$P < 0.01$).

Table 3. *Effect of dietary tin on iron retention by rats*

Parameter		Mean values† for rats fed: Tin-supplemented diet	Control diet
Kidney iron:	concentration (μg/g)	33·0 ± 5·3	30·6 ± 3·3
	content (μg/kidney)	26·9 ± 4·9	25·6 ± 2·7
Liver iron:	concentration (μg/g)	59·2 ± 11·9*	47·7 ± 5·9
	content (μg/liver)	561 ± 101	510 ± 67
Faecal iron:	days 7–10 (mg/day)	0·29 ± 0·04	0·32 ± 0·03
	days 14–17 (mg/day)	0·36 ± 0·04	0·37 ± 0·03

†Values are means ± 1 SD for groups of seven test and eight control rats. The control and test diets contained 1 and 206 μg tin/g, respectively. Test-group values marked with asterisks differ significantly (Student's *t*-test) from the corresponding values for the controls (*$P < 0.05$).

The rats fed the test diet lost 2·2 ± 0·5 (mean ± 1 SD) and 2·8 ± 0·3 mg tin/day in their faeces on days 7–10 and on days 14–17, respectively. The methods used in this study were not sensitive enough to monitor the loss of tin in the faeces of control animals. On average, the control animals consumed <15 μg tin/day, whereas the rats fed the tin-supplemented diet consumed about 2·4 mg tin/day on days 7–10 and 3·0 mg tin/day on days 14–17. The difference in the daily tin intake of rats during these two collection periods was due to the greater total food intake of the rats during the second period.

DISCUSSION

The zinc nutritional status, as indicated by the zinc levels in the tibias and kidneys, of the rats fed the tin-supplemented diet was poorer than in the control rats. The effect appeared to be due, at least partially, to impaired absorption of zinc rather than to depressed food intake. A common sign of zinc deficiency in rats is growth depression (Underwood, 1977). Several investigators have reported that the addition of large amounts of tin to the diet retarded growth in rats (de Groot, 1973; de Groot *et al.* 1973; Fritsch *et al.* 1977a). If the animals in the present study had been fed the same diet for a longer period of time or had been fed higher doses of tin, growth retardation due to a relative zinc deficiency probably would have occurred.

de Groot (1973) and de Groot *et al.* (1973) have reported that the anaemia observed among rats fed high levels of tin could be diminished or eliminated by copper supplementation of the diet. The copper nutritional status, as indicated by kidney- and liver-copper levels, of rats fed tin was depressed in this study. The addition of tin to the diet did not significantly affect faecal excretion of copper. Thus the effect of tin on the copper status of the rats may not have been caused by impaired absorption of copper. However, some coprophagy can occur even when rats are housed in wire-bottomed cages. Further studies need to be done with animals that do not practice coprophagy.

The increase in the iron concentration in the livers of rats fed the test diet may indicate an improvement in the iron nutritional status of the animals or an impairment in the animals' abilities to mobilize iron from the liver. Ceruloplasmin, a copper metalloenzyme, is one enzyme involved in the mobilization of iron from the liver. Perhaps the effect of tin on iron metabolism was a reflection of the effect of tin on copper metabolism.

Apparent faecal losses of ^{113}Sn by rats have been found to range from 90 to 100% of intake (Fritsch *et al.* 1977b; Hiles, 1974). In this study during the two collection periods, rats fed the test diet lost more than 90% of their total tin intake in the faeces. Although the rats absorbed little tin, the tibias and kidneys of the rats fed the test diet did accumulate more tin than the tissues of the control rats. Similarly Hiles (1974) observed that the amount of ^{113}Sn that accumulated in bone was approximately proportional to the oral dose of ^{113}Sn given to rats. Several investigators have

Table 4. *Effect of dietary tin on tin retention by rats*

Parameter		Mean values† for rats fed: Tin-supplemented diet	Control diet
Kidney tin:	concentration (μg/g)	1·5 ± 0·7***	0·2 ± 0·2
	content (μg/kidney)	1·3 ± 0·6***	0·2 ± 0·2
Tibia tin:	concentration (μg/g)	10·1 ± 3·3***	0·6 ± 0·6
	content (μg/tibia)	3·4 ± 1·2***	0·2 ± 0·2

†Values are means ± 1 SD for groups of seven test and eight control rats. The control and test diets contained 1 and 206 μg tin/g, respectively. Test-group values marked with asterisks differ significantly (Student's *t*-test) from the corresponding values for the controls (***$P < 0.001$).

reported that, of the soft tissues, kidney and liver are the most apt to accumulate tin (Benoy *et al.* 1971; Hiles, 1974; Schroeder *et al.* 1974).

Yamamoto, Yamaguchi & Sato (1976) found that the injection of tin acetate into rats caused elevated levels of calcium in the kidneys of those animals. The rats fed the test diet in this study did not accumulate more calcium in their kidneys than control rats. This may be because we fed low levels of tin, while Yamamoto *et al.* injected high levels of tin.

The addition of even moderate (206 μg/g) amounts of tin to the diet had an adverse effect on the zinc and copper nutritional status. It would be worthwhile to investigate whether the addition of more zinc and copper to the diets would overcome the adverse effects of dietary tin.

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VOLATILE NITROSAMINES IN SALT-PRESERVED FISH BEFORE AND AFTER COOKING

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Abstract—Uncooked, steamed and fried samples of salted fish were analysed for volatile nitrosamines by gas chromatography with detection by chemiluminescence and some were also analysed by combined gas chromatography and high-resolution mass spectrometry. *N*-Nitrosodimethylamine (NDMA) was detected in all of the samples that were analysed, whether cooked or uncooked. It was also detected in the aqueous phases derived from the steamed samples, and in the oil from frying in two out of three batches. In one of these the NDMA level was the highest encountered in the present study. *N*-Nitrosodiethylamine (NDEA), on the other hand, was detected in more batches of the steamed fish than of the uncooked or fried fish. It was not detected in the oil used for frying. In half of the steamed batches NDEA was detected only in the aqueous phases and in one batch it was detected only in the steamed fish. *N*-Nitrosodi-*n*-propylamine (NDPA) was detected in both the steamed and fried samples and *N*-nitrosodi-*n*-butylamine (NDBA) in only the fried samples. Neither was detected in the uncooked samples. *N*-Nitrosomorpholine (NMOR) occurred in the uncooked samples. Salted fish heads are sometimes used by southern Chinese to prepare soup. NDMA and NDEA occurred in both uncooked fish heads and in the soups prepared from them. The results confirm that certain nitrosamines are formed from precursors during cooking. A possible relationship between salted fish and certain cancers that are particularly prevalent among southern Chinese populations is discussed.

INTRODUCTION

Food has been studied as a potential source of nitrosamines for some years, and a review of these findings has been published by Scanlan (1975). Data based on mass-spectrometric measurements have been summarized by Gough (1978a). The vast majority of the studies have been undertaken on foods typical to the western world and to the UK in particular (Gough, Webb & Coleman, 1978).

Fish has been studied by several groups of workers and nitrosamines have been detected in smoked products as well as in fresh untreated fish. In most cases the levels were in the region of 10 μg/kg, but much higher amounts have occasionally been reported. Fong & Chan (1973 & 1976) have studied the occurrence of NDMA in Chinese marine fish treated with crude salt, which is normally used for preservation, and with pure sodium chloride. The levels of NDMA found were higher in the former (20 μg/kg) than in the latter (6 μg/kg). The crude salt used contained sodium nitrate as an impurity. The fish was found to harbour nitrate-reducing staphylococci which suggests that NDMA might have been produced in the salted fish as the result of an interaction between available secondary amines and nitrite in the fish. Formation *in vitro* of nitrosamines from their precursors by bacteria has been demonstrated by Collins-Thompson, Sen, Aris & Schwinghamer (1972), by Hawksworth & Hill (1971) and by Sander (1968).

Ho (1967 & 1975) studied the occurrence of nasopharyngeal carcinoma (NPC) in Hong Kong Chinese. The incidence rate for the cancer was found to be significantly higher among the people who lived and worked in boats and cooked in the open, than among the land-dwellers, who lived in congested dwellings with poor ventilation. Consequently, if an environmental factor was involved, it was likely to be an ingestant rather than an inhalant. Since the boat people usually consume far more salted fish than the land-dwellers, the feeding of salted fish from early childhood might be a major health risk (Ho, 1971, 1972 & 1975). This hypothesis is supported by the findings of an epidemiological case-control study carried out in Hong Kong by Geser, Charnay, Day, Ho & de Thé (1978) and by a study by Anderson, Anderson & Ho (1978) of the environmental backgrounds of 24 young Chinese NPC patients. In the former study multivariate analysis demonstrated that the consumption of salted fish during weaning was a risk factor in the development of NPC that was independent of traditional lifestyle which was itself a risk factor. In the latter study all 24 patients, 22 of whom were interviewed in the presence of their families, were found to have been fed salted fish from birth. Experimental data in support of the evidence for a role of salted fish in the aetiology of NPC were provided by Fong, Ho & Huang (1979), Ho, Huang & Fong (1978), Huang, Ho, Saw & Teoh (1978) and D. P. Huang *et al.* (unpublished data, 1979). Huang *et al.* (1978) induced

carcinomas in the nasal and paranasal regions of three out of 20 Wistar albino rats fed steamed salted fish as part of their diet for 12–24 months. No carcinomas were found in control rats fed rat chow only. In this study none of 15 rats given NDMA in their diet developed carcinoma, whereas three out of 14 given NDEA did. Fong *et al.* (1979) and Ho *et al.* (1978) demonstrated mutagenic activity in the urine of Wistar albino rats fed steam-cooked salted fish and found that the level of this activity decreased markedly when the rats were transferred from a salted fish diet to rat chow.

Earlier analyses for volatile nitrosamines carried out on uncooked salted fish (Huang, Ho, Gough & Webb, 1977) showed levels of NDMA no higher than those in some UK foods. The UK data have shown that cooking can influence the nitrosamine levels in foods by causing interactions between precursors. Because of this possibility and because cooked salted fish were used in the rat experiments, the volatile nitrosamine levels in uncooked salted fish were compared with those in the same fish prepared for consumption in the traditional Cantonese manner. The commonly available salted yellow croaker has been chosen in the present study.

Significant improvements have been made in analytical instrumentation for the detection of nitrosamines, the current detection limit being 2 ng/kg (Webb, Gough, Carrick & Hazelby, 1979).

EXPERIMENTAL

Materials. Salted yellow croaker was purchased on six different occasions from three different markets. Batch I was highly fermented, but the other batches (II–VI) were not. Heads of salted fish of several different species representative of the cheaper range sold in the markets were also purchased.

Procedure. Up to five samples were taken from each batch of fish, and each sample was homogenized and divided into three 250-g portions (A, B and C). Portion A was uncooked and was sent to London by express airmail delivery. Portion B was steam-cooked; the fish was placed on a porcelain dish supported by a stand above the boiling water level in a stainless-steel cooking utensil fitted with a lid. The fish was steamed for 15 min in about 1 litre of water, after which the exuded juice, together with the water vapour condensed on the dish were separated from the solid matter by decanting. The resultant fluid and the fish were immediately airmailed to London. Portion C was fried in an open pan for 10 min in boiling peanut oil, the oil commonly used locally. After frying, the fish and the oil were separated and despatched to London.

Three batches of salted fish heads were analysed without any pretreatment. A further three batches were used to prepare soup by boiling 200 g of fish heads in 250 ml water for 30 min in a closed container. Analysis was carried out on the aqueous concentrate. In the animal feeding experiment carried out by Huang *et al.* (1978) the rats were fed such a soup diluted 1:5 with tap water.

Preparation of all of the samples for nitrosamine analysis followed standard practice (Goodhead & Gough, 1975) by adding 250 ml water and 100 g NaCl to 250 g salted fish and steam-distilling the resultant mixture. To 400 ml of the distillate 80 g NaCl and 4 ml 10 N-sulphuric acid were added and the mixture was extracted four times with 40 ml dichloromethane. The combined extracts were washed with 70 ml 1·5 N-NaOH, separated and dried over sodium sulphate. The volume of the extract was reduced by careful evaporation at 45°C to 250 μl and 5-μl aliquots were analysed. This procedure gives recoveries between 60 and 80% depending on the particular nitrosamine. The frying oil and the aqueous phase from the steaming were extracted without distillation and then treated as above. Analysis of the extracts was by gas chromatography with detection by chemiluminescence. Details of this technique have been previously published (Gough, 1978b). In summary, the constituents of the extracts are separated on a polar chromatographic column at 150°C and pass into a catalytic chamber at 450°C. Nitrosamines present are cleaved at the N–NO bond and the nitric oxide so formed is passed into a reaction chamber containing ozone. Interaction between ozone and nitric oxide gives an infra-red emission which is detected by a photomultiplier tube. Over the range of concentrations studied, the response to nitric oxide is linear and since generation of nitric oxide, at least from the nitrosamines under study, is quantitative, the amount of nitric oxide detected is directly related to the concentration of nitrosamine present in the extract (Gough, Webb & Eaton, 1977). Several of the extracts were also examined by combined gas chromatography and high-resolution mass spectrometry using the methods previously described (Gough & Webb, 1972; Webb *et al.* 1979). The chemiluminescence detector will respond to any *N*-nitroso compound that passes through the gas chromatograph. Unlike the mass spectrometer, detection is not limited to certain specific nitrosamines, which in the previous study (Huang *et al.* 1977) were four dialkyl nitrosamines, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine. The chemiluminescence detector does respond to compounds other than *N*-nitroso compounds, but a comparison of chemiluminescence and mass-spectrometric data has shown that false positive results by chemiluminescence are not often encountered in practice (Webb *et al.* 1979). In the present study the eight samples examined by combined gas chromatography and mass spectrometry gave results in agreement with chemiluminescence data in all cases.

RESULTS

NDMA was detected in all of the samples of all of the batches of yellow croaker, whether uncooked or cooked. For the different samples within each similarly treated batch the levels were in excellent agreement, and only the mean values are quoted. These are given in Table 1. Within a given batch, there was very little difference between the levels in the uncooked, steamed and fried fish, except that in four out of six batches NDMA levels in the fried fish were two or more times higher than those of the uncooked fish of the same batch.

NDMA was also detected in all aqueous-phase fractions derived from the steamed batches and in the oil after frying in two out of three batches tested. In

one of these the NDMA level was the highest encountered in the present study. The highly fermented fish (batch I) did not have a higher NDMA level than the other batches.

N-Nitrosodiethylamine (NDEA) was detected in the uncooked fish in batches II and VI (Table 2). It occurred in several batches of steamed fish, but in only one batch of fried fish. When it did occur, it was present in all of the samples of the batch. It was also detected in the aqueous phase from the steamed fish, but not in the oil from frying.

Table 3 shows the occurrence of other volatile nitrosamines: *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosodi-*n*-butylamine (NDBA) and *N*-nitrosomorpholine (NMOR). NDPA was detected in the cooked samples of one batch but not in the uncooked. NDBA was found in the same batch after frying. NMOR, which was detected in the uncooked fish of a further batch, did not survive cooking or frying. The untreated fish heads all contained NDMA and one batch also contained NDEA (Table 4). The fish-head soups all contained NDMA and one also contained NDEA.

DISCUSSION

Because these nitrosamines are volatile and frying was in an open pan and at a higher temperature than

Table 1. N-*Nitrosodimethylamine (NDMA) concentrations in uncooked, steamed or fried salted yellow croaker*

	NDMA concentration in				
		Steamed		Fried	
Batch (no. of samples)	Uncooked fish (ng/kg)	Fish (ng/kg)	Aqueous phase (ng/litre)	Fish (ng/kg)	Oil (ng/litre)
I (3)	300*	500	200	200*	ND
II (3)	500*	600	300	1400	2700
III (3)	600*	600	400	400*	1300
IV (5)	100	200	400	400*	NT
V (5)	100	100*	100	200	NT
VI (5)	100	100	300	200	NT

ND = Not detected NT = Not tested

* Confirmed by high-resolution mass spectrometry.

The total number of analytical determinations carried out was 170.

Table 2. N-*Nitrosodiethylamine (NDEA) concentrations in uncooked, steamed or fried salted yellow croaker*

	NDEA concentration in				
		Steamed		Fried	
Batch (no. of samples)	Uncooked fish (ng/kg)	Fish (ng/kg)	Aqueous phase (ng/litre)	Fish (ng/kg)	Oil (ng/litre)
I (3)	ND	100	ND	ND	ND
II (3)	50	60	ND	ND	ND
III (3)	ND	60	ND	ND	ND
IV (5)	ND	60	90	ND	NT
V (5)	ND	30	50	10	NT
VI (5)	20	ND	200	ND	NT

ND = Not detected NT = Not tested

The total number of analytical determinations carried out was 170.

Table 3. *Concentration of other volatile nitrosamines in salted yellow croaker*

		Concentration of nitrosamine in				
			Steamed		Fried	
Volatile nitrosamine	Batch (no. of samples)	Uncooked fish (ng/kg)	Fish (ng/kg)	Aqueous phase (ng/litre)	Fish (ng/kg)	Oil (ng/litre)
NDPA	V (5)	ND	50	ND	30	NT
NDBA	V (5)	ND	ND	ND	50	NT
NMOR	VI (5)	200	ND	ND	ND	NT

NDPA = *N*-Nitrosodi-*n*-propylamine NDBA = *N*-Nitrosodi-*n*-butylamine
NMOR = *N*-Nitrosomorpholine ND = Not detected NT = Not tested

The total number of analytical determinations carried out was 170.

Table 4. *Concentrations of N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) in fish heads and in fish-head soup*

Batch	Concentration of nitrosamines in Uncooked fish heads (ng/kg)	Fish-head soup (ng/litre)
	NDMA	
1	80*	60
2	60	10
3	100	60
	NDEA	
1	ND	ND
2	ND	40
3	100	ND

ND = Not detected

* Confirmed by mass spectrometry.

steaming, it is surprising that there were any nitrosamines still present even though they may be formed during the frying process. It has been shown that 90% of NDMA could be lost to the atmosphere during the frying of bacon (Gough, Goodhead & Walters, 1976). The amounts detected in the frying oil in the present work are very likely to be a fraction of the amount actually present during frying. Furthermore, there must have been some NDMA formation during frying since there was more NDMA detected after frying than in the uncooked fish of the same batch. When uncooked, batch II contained 500 ng NDMA/kg fish. After frying the fish itself contained 1400 ng/kg, and the concentration of NDMA in the oil (2700 ng/litre) represented a further 500 ng NDMA/kg fish without allowing for possible substantial losses due to evaporation. In the absence of fish the oil did not contain any nitrosamines. Oil is usually used repeatedly for cooking, and hence the nitrosamine level is likely to rise with use despite losses by heating.

In the evaluation of the carcinogenic effect of NDMA, NDEA, NDPA, NDBA and NMOR, an IARC Working Group (1978) found that there was sufficient evidence of such an effect for all of them in several species of experimental animals. Although no epidemiological data were available, the Working Group concluded that the compounds should be regarded for practical purposes as if they were carcinogenic to humans. Therefore it is possible that salted fish consumption may be a risk factor in the development of those cancers (e.g. NPC and hepatocellular carcinoma) that are particularly prevalent among the southern Chinese. The respiratory tract and the liver have been shown to be vulnerable to the carcinogenic effect of those nitrosamines detected in the cooked salted fish in the present study. It is also possible that the cooking methods used by southern Chinese may play a part in producing or accentuating the risk.

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ANALYSIS OF 1-METHYL-1,2,3,4-TETRAHYDRO-β-CARBOLINE IN ALCOHOLIC BEVERAGES

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Abstract—A gas chromatographic–mass spectrometric method is described for the analysis of 1-methyl-1,2,3,4-tetrahydro-β-carboline in alcoholic beverages. The analytical procedure involves the addition of semicarbazide and a deuterated analogue as the internal standard, followed by extraction with toluene–isoamyl alcohol and derivatization with pentafluoropropionic anhydride. Multiple ion detection showed 1-methyl-1,2,3,4-tetrahydro-β-carboline to be present in alcoholic beverages prepared by fermentation processes. The range of concentrations was 4·8–450 nmol/litre in wine and 9·1–69 nmol/litre in beer.

INTRODUCTION

Among congeners in alcoholic beverages, β-carbolines have previously attracted some attention because of the identification of harman (1-methyl-β-carboline) in wine and saké (Lashkhi & Mudzhiri, 1975; Takase & Murakami, 1966). The existence of this compound may be of significance for chemical carcinogenesis, since it potentiates the action of mutagens in bacterial test systems (Sugimura, 1979). Other effects of β-carbolines are monoamine oxidase inhibition (Buckholtz & Boggan, 1977), monoamine reuptake inhibition (Rommelspacher, Strauss & Rehse, 1978) and the stimulation of potassium-induced release of serotonin from brain slices (Rommelspacher & Subramanian, 1979). It has also been reported that intraventricular administration of a β-carboline to rats increases the voluntary intake of ethanol (Melchior & Myers, 1977). On the other hand, on repeated peripheral administration, this β-carboline has an inhibitory effect on liver alcohol dehydrogenase activity and reduces ethanol intake in rats (Messiha, Larson & Geller, 1977).

The tetrahydro-β-carbolines are formed readily by reaction of tryptamines with carbonyl compounds and subsequent intramolecular cyclization of the resulting Schiff base. This sequence is known as the Pictet–Spengler reaction and takes place *in vitro* under quasi-physiological conditions of temperature and pH (Whaley & Govindachari, 1951). Since acetaldehyde is formed during the fermentation process, and simple decarboxylation of the amino acid tryptophan yields tryptamine, formation of 1-methyl-1,2,3,4-tetrahydro-β-carboline (1MTHBC) might be suspected. The present report is devoted to the identification and quantitation of 1MTHBC in alcoholic beverages using a gas chromatographic–mass spectrometric method.

EXPERIMENTAL

Materials. Tryptamine hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO), indole from Aldrich Chemical Co. (Beerse, Belgium), pentafluoropropionic anhydride from Massanalys AB (Bromma), lithium aluminium deuteride from Fluka AG (Buchs, Switzerland), and deuterium oxide and deuterium chloride from E. Merck AG (Darmstadt, FRG). All other chemicals were of analytical purity.

The synthesis of [α,α,β,β-$^2H_4$]tryptamine HCl ([$^2H_4$]T) from indole was performed according to a scheme developed for the synthesis of other tetradeuterated tryptamines (Shaw, Wright & Milne, 1977). The synthesis of 1-methyltetrahydro-β-carboline hydrochloride from tryptamine and acetaldehyde was carried out according to Akabori & Saito (1930). Similarly 1-methyl-[3,3,4,4-$^2H_4$]tetrahydro-β-carboline hydrochloride was prepared from [$^2H_4$]T. Treatment of [$^2H_4$]1MTHBC with 5% ^2HCl in ^2H_2O for 1 hr at 80°C yielded 1-methyl-[3,3,4,4,5,6,7,8-$^2H_8$]-tetrahydro-β-carboline ([$^2H_8$]1MTHBC) as a precipitate after addition of NaOH. The mass spectra of 1MTHBC, [$^2H_4$]-1MTHBC and [$^2H_8$]1MTHBC as pentafluoropropionyl (PFP) derivatives are shown in Fig. 1.

The alcoholic beverages were obtained from AB Vin- & Spritcentralen, Stockholm, and Pripps AB, Stockholm. The seals on all bottles were broken immediately before analysis.

Preparation of samples. Aliquots (0·5 ml) of the beverage samples were pipetted into 15-ml glass-stoppered test tubes containing 0·5 ml distilled water, 4·0 mg semicarbazide, 49·1 pmol [$^2H_8$]1MTHBC, 508 pmol [$^2H_4$]T 0·4 g NaCl, 0·1 ml 10 M-NaOH and 5 ml 3% isoamyl alcohol in toluene. The tubes were shaken for 10 min, and then centrifuged at 1000 *g* for 5 min. The organic layers were transferred to clean tubes and evaporated to dryness under a stream of nitrogen. The residues were derivatized by treatment with 50 μl PFPA and 50 μl benzene at 60°C for 20 min. After cooling, the excess reagents were evaporated under a stream of nitrogen. The residues were redissolved in 20 μl ethyl acetate.

Stock solutions of 1MTHBC were prepared in water and stored in the dark at 4°C. From the stock solutions, standard samples were derived as described above for the alcoholic beverage samples.

Gas chromatography–mass spectrometry. A computer-controlled LKB 2091 gas chromatograph–mass spectrometer was used both for the multiple ion detection and the recording of mass spectra. The gas-chromatographic separations were achieved using a

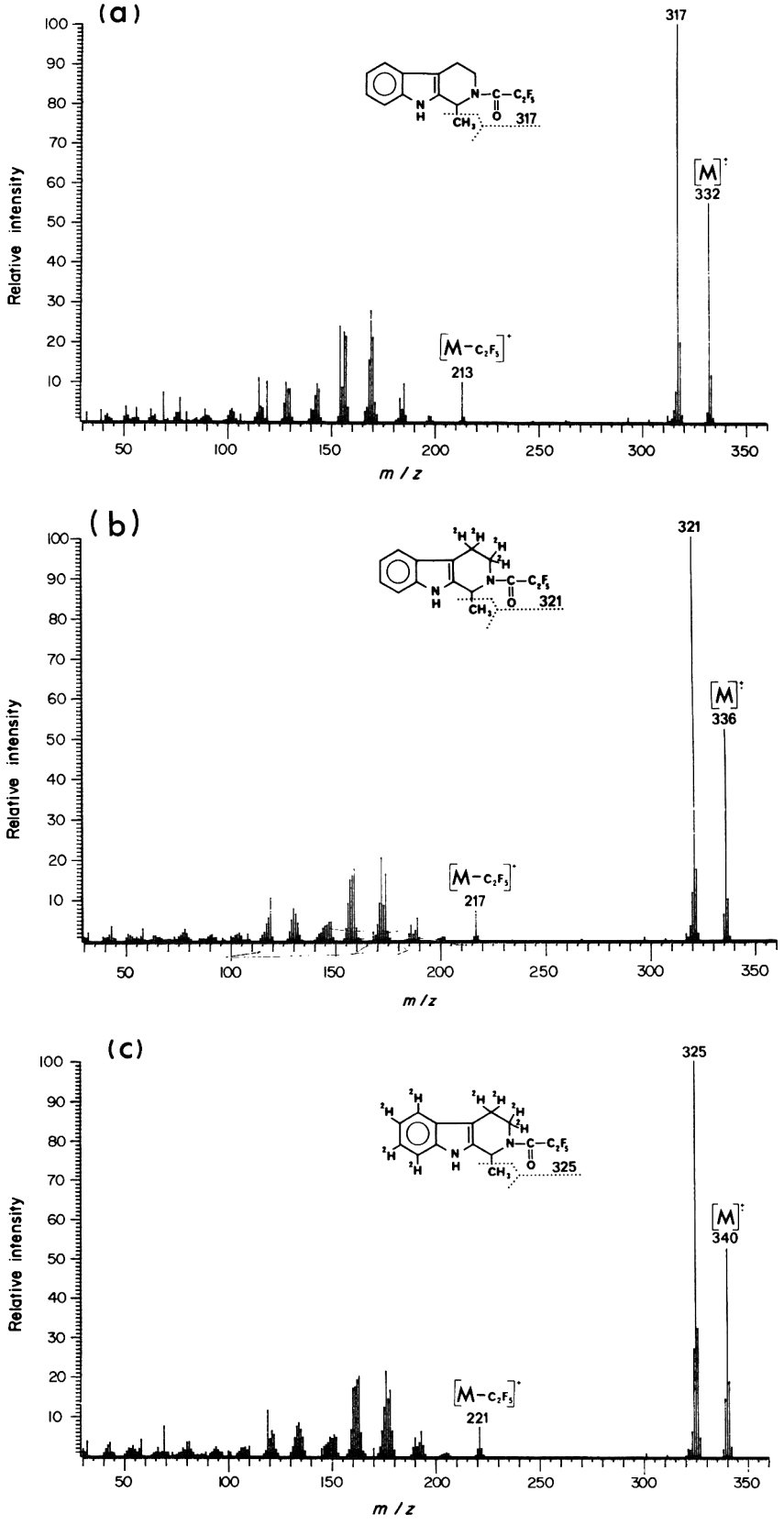

Fig. 1. Electron-impact mass spectra of (a) the pentafluoropropionyl derivative of 1-methyl-1,2,3,4-tetrahydro-β-carboline (1MTHBC–PFP), (b) [$^2H_4$]1MTHBC–PFP and (c) [$^2H_8$]1MTHBC–PFP.

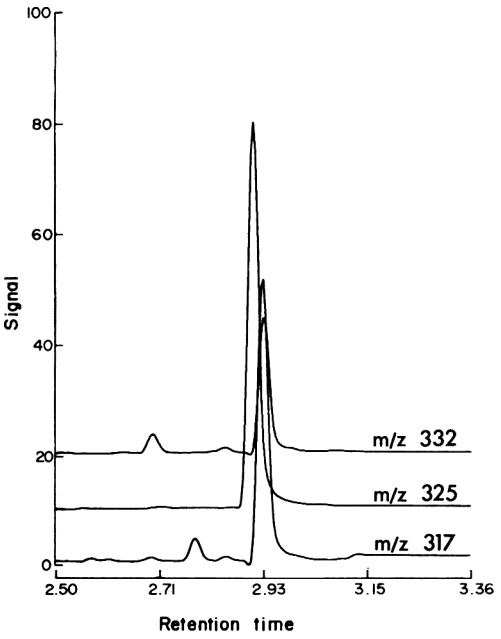

Fig. 2. Chromatogram obtained in the analysis of Pripps non-alcoholic beer. Mass numbers are indicated on the figure; the relative gain amplification factors were the same on all channels.

20 m SE 52 WCOT glass capillary column (ID 0·25 mm). Helium was used as a carrier and make-up gas. Splitless injections were carried out using a 'moving needle' device. The gas-chromatographic conditions were: injector heater 250°C, column temperature 230°C, column flow rate c. 2 ml/min and make-up gas flow rate *c.* 12 ml/min. Aliquots (2 μl) of the samples were injected and the valve was opened after an initial delay of 2·5 min to avoid contamination of the ion source. Under these conditions, the retention time of 1MTHBC–PFP was approximately 2·9 min. The mass-spectrometric conditions were: separator temperature 240°C, ion source temperature 240°C, electron energy 70 eV and trap current 50 μA.

Quantitation. Calibration curves were constructed by plotting the peak height ratios (*m/z* 332/325) of the standard samples against the 1MTHBC concentration. The 1MTHBC levels were determined from the peak height ratios of each sample by reference to the calibration curve.

RESULTS AND DISCUSSION

The electron-impact mass spectra of the PFP derivatives of 1MTHBC (Fig. 1a), [$^2H_4$]1MTHBC (Fig. 1b) and [$^2H_8$]1MTHBC (Fig. 1c) confirm the presence of one PFP group per molecule. The compounds show abundant molecular ion at *m/z* 332, 336 and 340, respectively. The base peaks at *m/z* 317, 321 and 325 result from loss of the methyl group.

Multiple ion detection was performed by monitoring the ion intensities at *m/z* 317, 325 and 332. The chromatograms obtained from the analysis of extracts (Fig. 2) show that a compound was present possessing both the same retention time and relative ion intensity as authentic 1MTHBC. The possibility of formation of 1MTHBC during sample preparation was checked by the addition of [$^2H_4$]T to the samples and analysis of the extracts for [$^2H_4$]1MTHBC by monitoring ion intensities at *m/z* 336. No evidence for [^2H]1MTHBC formation was obtained. If, however, the addition of semicarbazide to trap carbonyl compounds was omitted, [$^2H_4$]1MTHBC could be detected in the extracts.

1MTHBC was determined quantitatively from the peak height ratios *m/z* 332/325. A typical calibration curve is shown in Fig. 3. The lower limit of detection was estimated to be *c.* 0·1 pmol in a sample. The analyses of 1MTHBC in various alcoholic beverages showed that the compound was detectable in those prepared by fermentation but not in pure distillates (Tables 1 & 2). The concentration of 1MTHBC in wine was found to cover a rather wide range (4·8–450 nmol/litre). In beer, however, the range of concentrations was narrower (9·1–69 nmol/litre).

The origin of the 1MTHBC in the alcoholic beverages is not known. One possibility is that tryptamine and acetaldehyde react non-enzymatically (Whaley & Govindachari, 1951). Other ways are also possible but would require enzymatic assistance. In addition, β-carbolines (harman alkaloids) occur naturally in some plants (Allen & Holmstedt, 1980) and 1MTHBC may be present in the raw materials used for manufacturing alcoholic beverages.

Recently the endogenous occurrence of several β-carbolines in mammalian tissue has been reported (Barker, Harrison, Brown & Christian, 1979; Shoemaker, Cummins, Bidder, Boettger & Evans, 1980). It has been suggested that the formation and action of β-carbolines after ethanol consumption may play a role in the aetiology of alcoholism. Obviously, the presence of 1MTHBC in alcoholic beverages must be considered when its possible role in alcoholism is being elucidated. Many pertinent questions, like the absorption of 1MTHBC from the intestine and its metabolic fate, remain to be investigated.

The presence of 1MTHBC in alcoholic beverages demonstrated in this study may be of significance because tetrahydro-β-carbolines have been shown to

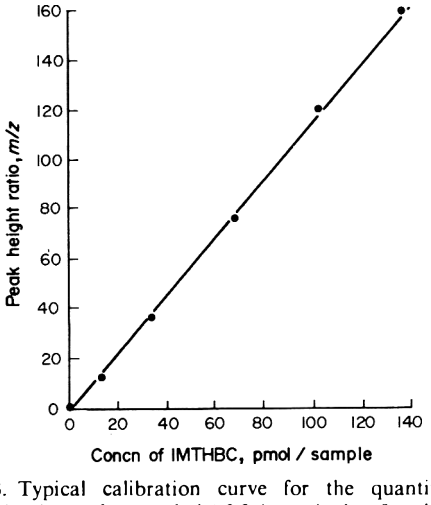

Fig. 3. Typical calibration curve for the quantitative determination of 1-methyl-1,2,3,4-tetrahydro-β-carboline (1MTHBC).

Table 1. *Concentrations of 1-methyl-1,2,3,4-tetrahydro-β-carholine (1MTHBC) in various alcoholic beverages*

Beverages	Country of origin	Concn of 1MTHBC (nmol/litre)
Spirits		
Aquavite	Sweden	<0·25
Aguardiente Chuchuhuasi*	Peru	<0·25
Red wines		
Medoc	France	66
Chianti Rosso	Italy	214
Parador	Spain	80
Bourgogne Vieux	France	450
Turenne	France	136
White wines		
Liebfraumilch	Germany	426
Maritime	France	6·5
Zeller schwarze Katze	Germany	231
Beyaz	Turkey	4·8
Val de Loire	France	31
Miscellaneous		
Sherry Manzanilla	Spain	41
Sherry Manzanilla la Capitana	Spain	104
Sakė	Japan	394

*Locally distilled aquavite.

Table 2. *Concentrations of 1-methyl-1,2,3,4-tetrahydro-β-carboline (1MTHBC) in various types of beer*

Beer*	Alcohol content (%, w/w)	Concn of 1MTHBC (nmol/litre)
Pripps non-alcoholic	—	37
Carlsberg non-alcoholic	—	16
Pripps Blå Lättöl	1·8	27
Falcon	1·8	9·1
Pripps Blå Extra	2·8	33
Falcon Gold Extra	2·8	15
Pripps Jubileum Export	4·5	69
Golden Crown Falcon	4·5	45

*All from Sweden except Carlsberg non-alcoholic, which is from Denmark.

influence certain mechanisms in the central nervous system. In addition, it should be pointed out that 1MTHBC can serve as a precursor of harman, a comutagen (Sugimura, 1979).

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LONG-TERM EFFECTS OF FEEDING AFLATOXIN-CONTAMINATED MARKET PEANUT OIL TO SPRAGUE–DAWLEY RATS

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(*Received 24 March 1980*)

Abstract—Peanut oils obtained from Hong Kong markets were frequently contaminated with aflatoxins. A 'purified' diet in which aflatoxin-contaminated market peanut oil (aflatoxin B_1 110 ppb [μg/kg]) was used as the fat source was fed to Sprague–Dawley rats for 22 months from weaning. Its estimated aflatoxin B_1 content was 5–7 ppb. Controls were fed a diet of identical composition except that Mazola corn oil (aflatoxin-free) was used. At autopsy, three out of 76 aflatoxin-fed rats were found to have sarcomas—one in the liver, one in the wall of the colon and one in the subcutaneous tissue of the groin. Eighteen animals fed peanut oil showed parenchymal liver damage and varying degrees of fatty change and one showed pre-malignant changes in liver cells. Of 90 control rats, none developed malignant tumours. The liver-to-body weight ratios for experimental and control rats were 2·93 ± 0·46 and 2·62 ± 0·58, respectively (means ± 1 SD). The difference between these values was statistically significant ($P < 0{\cdot}01$), reflecting the degree of fatty change in the livers of experimental rats compared with that in the controls. Over 90% of Hong Kong households use peanut oils for cooking purposes, and these data appear to indicate a possible health hazard in the use of peanut oils contaminated at the levels found.

INTRODUCTION

Aflatoxin has been implicated as an aetiological factor in human primary liver-cell cancer in various high-risk areas of Africa and Asia. Alpert, Hutt, Wogan & Davidson (1971) found in Uganda that the aflatoxin contamination of foods was greatest in areas with the highest incidence of this cancer. A similar situation was reported by Shank, Bhamarapravati, Gordon & Wogan (1972a) in Thailand. Though final proof is lacking, these findings indicate that the link between areas where liver-cell cancer is endemic may be aflatoxin.

The incidence of primary liver cancer is high also in Hong Kong, the age standardized rate being 35·6 per 100,000 per year for males (Cancer Registry, 1976), but aflatoxin contamination of foods and foodstuffs has been found to be low. Of 878 samples analysed by Shank, Wogan, Gibson & Nondasuta (1972c), only 22 contained these mycotoxins. However, peanut oil was not tested in that study and since peanut oil is used daily in over 90% of the local Chinese households for cooking purposes, it could be the most likely source of aflatoxin for the local people. In a recent paper, Fong, Ton, Koonanuwatchaidet & Huang (1980) reported that peanut oils obtained from local markets were frequently contaminated with aflatoxins, with levels reaching 98–150 ppb (μg/kg) in three of the samples analysed.

The investigation reported here used Sprague–Dawley rats to determine whether prolonged ingestion of such aflatoxin-contaminated peanut oil, consumed as a fat component of the daily diet, would constitute any hazard.

EXPERIMENTAL

Composition of 'purified' diets. The components of the diets were as follows: casein (220 g/kg), methionine (3 g/kg), cornstarch (320 g/kg), sucrose (344 g/kg), vegetable oil (Mazola corn oil or peanut oil; 55 g/kg), mineral mix (35 g/kg), vitamin mix (20 g/kg), choline chloride (3 g/kg) and vitamin B_{12} (50 μg/kg). The mineral mix contained the following (in g): $CaCO_3$ (190·5), $CaHPO_4$ (144·1), K_2HPO_4 (403·8), $MgCO_3$ (73·2), $MgSO_4.7H_2O$ (191·7), $FeSO_4.7H_2O$ (17·4), $MnSO_4.H_2O$ (15·4), KI (0·02), $CuSO_4.5H_2O$ (1·96), NaF (0·22), NaCl (82·4), Na_2CO_3 (40·6), $Cr_2(SO_4)_3.H_2O$ (0·009), Na_2SeO_3 (0·02), $ZnSO_4$ (3·8) and glucose (1041). The components of the vitamin mix (in g) were menadione (0·1), riboflavin (0·4), thiamine HCl (0·8), pyridoxine (0·8), calcium pantothenate (2), folic acid (1), nicotinic acid (5), inositol (25), Roche A & D mix, 500/50 (6), dl-α-tocopherol acetate, 500 U/g (22·5) and sucrose (1936·8).

Vegetable oils used in diets. Peanut oils obtained from local markets were analysed for aflatoxin contamination. Three batches of a popular brand were found to contain aflatoxin B_1 at levels of 98, 110 and 125 ppb, as determined by the traditional chemical method (Association of Official Analytical Chemists, 1975). Mutagenic activity was also demonstrated in these samples (Fong *et al.* 1980) by the Ames Salmonella/mammalian-microsome mutagenicity assay (Ames, McCann & Yamasaki, 1975). These oils were pooled and used as a source of vegetable oil in the diet of the group A rats (see below). As the vegetable oil concentration in the diet was 5·5%, the level of aflatoxin B_1 contamination in diet A was initially

6·2 ppb. Samples of diet A were analysed periodically. and aflatoxin B_1 levels were found to be between 5 and 7·5 ppb. Mazola corn oils which had no detectable mutagenic activity and were aflatoxin-free (Fong *et al.* 1980) were used as the source of vegetable oil for the group B rats.

Animals and diet. Sprague–Dawley rats were obtained from the Laboratory Animal Unit, University of Hong Kong, and were maintained under SPF conditions. Male and female weanlings (mean body weight 42 ± 5 g) were divided randomly into two groups (A and B), each comprising 60 males and 30 females. They were housed five to a cage and were given diet and water *ad lib.* Group A rats were given the diet containing contaminated peanut oil as a source of fat, while group B rats were given that containing Mazola corn oil. The animals were weighed every fortnight. Food consumption was measured daily for each cage. Some animals were killed during the course of the experiment because of their poor condition, and all survivors were killed at 22 months (95 wk). Complete autopsies were performed on all these animals. The liver, spleen, kidney, lung, stomach, intestine, bladder and testis were fixed in buffered formalin and examined histologically. Several animals that died during the study were unfit for autopsy when found.

RESULTS

Body weights in the two groups were comparable throughout the study and there were no significant differences between the levels of consumption of the Mazola-oil and peanut-oil diets. In group A (fed peanut oil) 14 rats died at scattered intervals during the experiment and were not examined. Of the 76 group-A rats autopsied, 18 showed parenchymal liver damage and varying degrees of fatty change. The damaged liver cells were swollen and the cytoplasm was granular. One of these rats was found to have a lesion that was considered to be pre-malignant; the cells were characterized by an increase in the nuclear/cytoplasmic ratio, hyperchromatic nuclei and multiple nucleoli (Fig. 1a), while the uninvolved cells at the periphery were compressed (Fig. 1b). A few of the 90 group-B (control) animals showed mild fatty changes in the liver (Fig. 2). The ratios of liver weight to body weight for the experimental and control rats were 2·93 ± 0·46 and 2·62 ± 0·58 (means ± 1 SD), respectively. The difference between these values was statistically significant ($P < 0·01$ by Student's t test) and reflected the greater degree of fatty change in the livers of the experimental rats as compared with the controls. Within each group, there was no difference in this ratio between male and female rats. No Taenia infestation was found in any of the animals used in this study.

Five group-B and four group-A rats developed benign mammary tumours. Three of the males in group A were found to have sarcomas, one in the wall of the colon, one in the liver and one in the subcutaneous tissue of the groin. The two latter tumours were in rats killed at month 18. None of the group-B rats developed malignant tumours, and in fact we have never found any spontaneous malignant tumours in our control rats.

The liver sarcoma showed marked pleomorphism. Many cells were multinucleate (Fig. 3) and there was abundant cytoplasm. Other cells in the tumour were spindle-shaped. The histogenetic origin is uncertain, but it is possible that the tumour was derived from histiocytes. The colonic tumour was a leiomyosarcoma, with plump, spindle-shaped tumour cells and prominent mitotic activity. The subcutaneous sarcoma in the groin was cellular, with small, spindle-shaped cells and abundant intercellular mucin. Tumour necrosis was prominent.

DISCUSSION

Aflatoxin B_1 has been shown to induce liver cancer in a wide range of animals (Butler, 1969; Heathcote & Hibbert, 1978) and at doses lower than any other hepatocarcinogen (Wogan, Paglialunga & Newberne, 1974). The latter authors reported a 9% incidence of liver cancer in Fischer rats exposed continuously to a diet containing 1 ppb aflatoxin B_1. The present investigation showed a lower incidence with a higher dietary level of aflatoxin B_1; a 1·3% liver-cancer incidence or a 5% total tumour incidence was observed in rats of the Sprague–Dawley strain exposed continuously to a diet containing 5–7 ppb aflatoxin B_1 derived from peanut oil. This difference may be due to the considerable variation in susceptibility between species and strains. Fischer rats have been reported to be more susceptible to this carcinogen than rats of the Sprague–Dawley strain (Wogan *et al.* 1974). Also, in the present study, only parenchymal damage and one pre-malignant nodule were demonstrated in the liver. The only true tumour found in the liver was a sarcoma. This may be due to the very low doses administered and the particular strain used in our study.

On the other hand, it would be helpful to calculate the local population's average daily intake of aflatoxin B_1 in peanut oils. Most Chinese dishes are 'fried' or cooked with peanut oils, and the average daily intake of peanut oils per person on a family basis was estimated to be about 50–60 g. This would give an average daily intake of aflatoxin B_1 from peanut oil alone of about 0·85–8·5 μg or 13–133 ng/kg body weight, assuming a 15–150-ppb level of aflatoxin B_1 contamination in peanut oils sold in local markets (Fong *et al.* 1980). In high-risk areas in Thailand and Mozambique, the average daily intake of aflatoxins was 45 ng/kg body weight (Shank, Gordon, Wogan, Nondasuta & Subhamani, 1972b) and 222 ng/kg (van Rensburg, van der Watt, Purchase, Coutinho & Markham, 1974), respectively. There are no reports of a low frequency of liver-cell cancer in areas of high aflatoxin exposure. Our calculations, based on aflatoxin B_1 in peanut oils alone, are compatible with those in the literature. Other frequently contaminated local foodstuffs, such as dried lotus seeds (Shank *et al.* 1972c) and peanuts (Fong *et al.* 1980) were eaten occasionally, as snacks. Therefore, it is difficult to assess their share in the total daily aflatoxin ingestion.

Unless the susceptibility of the human liver to this carcinogen is very different from that of the rat, our data suggest a definite hazard in the ingestion of peanut oils contaminated with 110 ppb aflatoxin B_1. Comparison of our calculations on the average daily ingestion of aflatoxins by the local population with

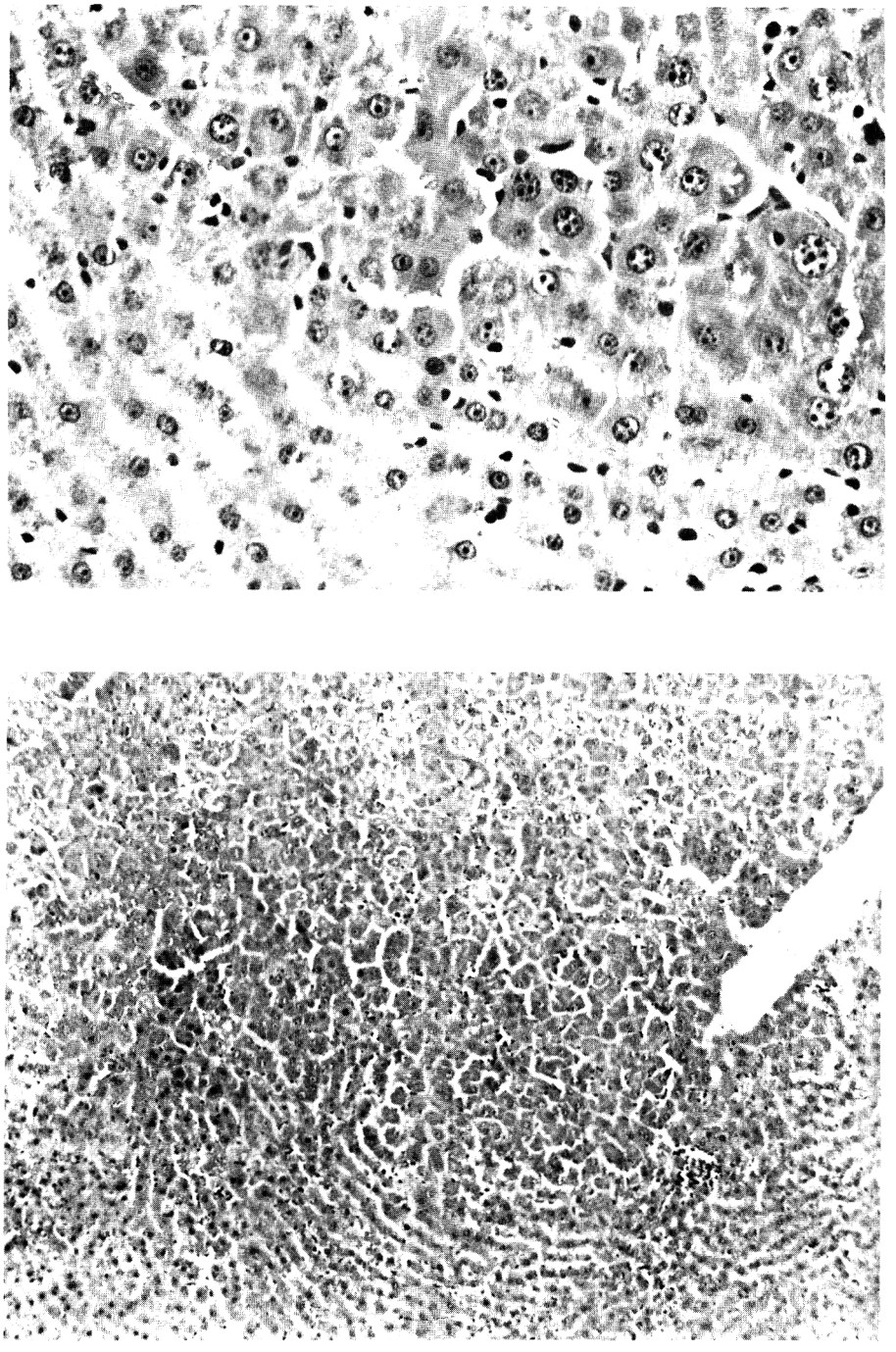

Fig. 1. Pre-malignant liver lesion in male rat fed aflatoxin B_1-contaminated peanut oil in the diet for 22 months, showing (a) cells with large hyperchromatic nuclei and multiple nucleoli and (b) the pre-malignant nodule compressing the surrounding liver cells. Haematoxylin and eosin $\times 400$ (a) and $\times 110$ (b).

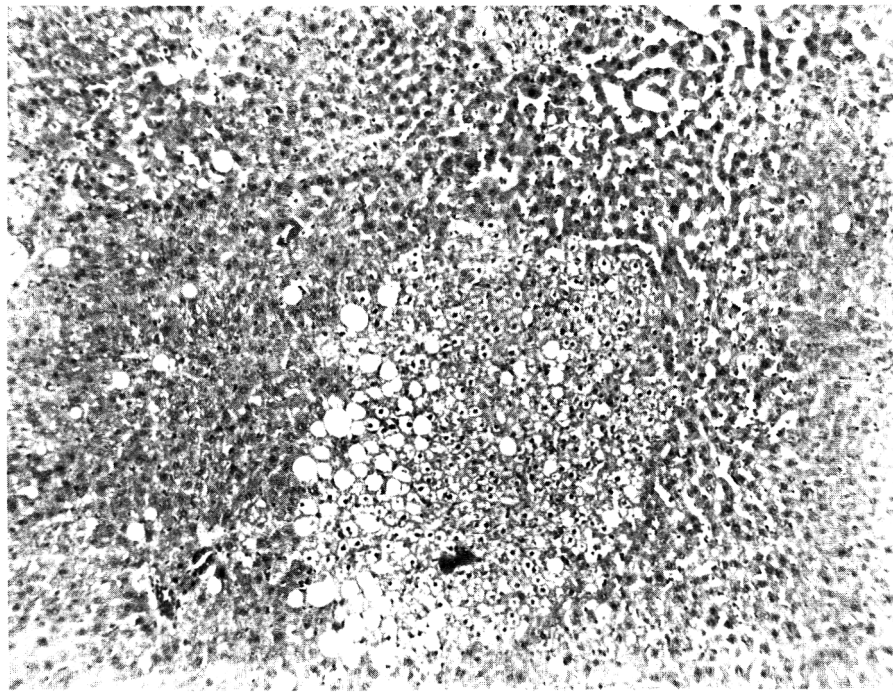

Fig. 2. Fatty degeneration of the liver of a control rat, showing marked vacuolation of the cytoplasm in the liver cells of one area. Haematoxylin and eosin ×95.

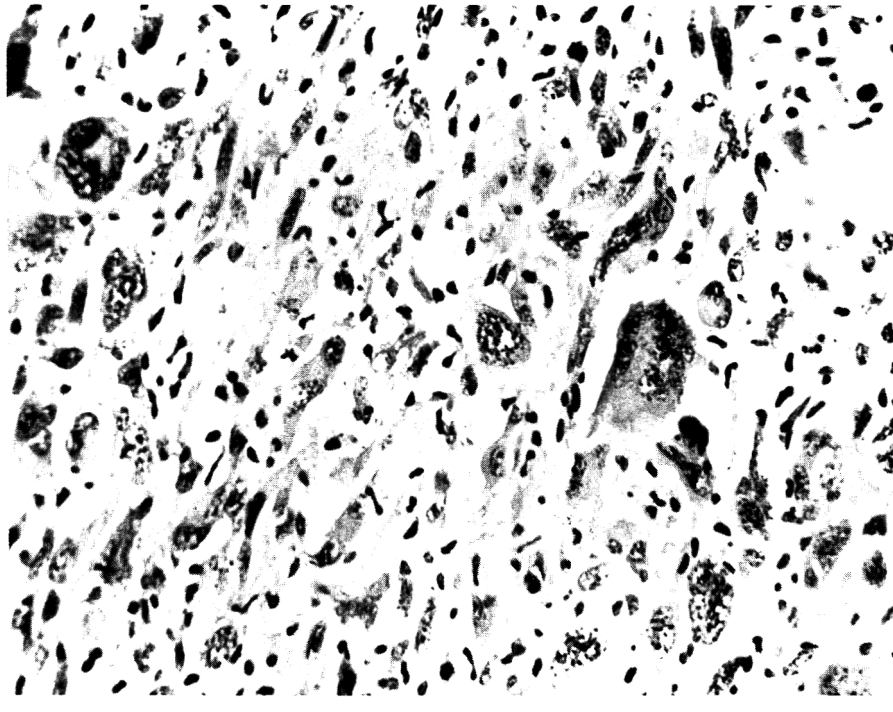

Fig. 3. Sarcoma in the liver of a male rat fed aflatoxin B_1-contaminated peanut oil in the diet for 18 months, showing multinucleate tumour cells, spindle cells and marked nuclear pleomorphism. Haematoxylin and eosin ×364.

those in the literature also support such a hypothesis. Naturally, the association between cancer incidence and carcinogen-intake is not equivalent to a cause/effect relationship. The action of the carcinogen may be dependent on interactions with agents such as alcohol and viral hepatitis.

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THE TOXICITY OF T-2 TOXIN AND DIACETOXYSCIRPENOL IN COMBINATION FOR BROILER CHICKENS

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Abstract—T-2 toxin and diacetoxyscirpenol, two naturally occurring trichothecene mycotoxins, were dissolved in dimethylsulphoxide:saline (1:9, v/v) and administered separately and in combinations to 7-day-old male broiler chickens. The 72-hr single oral dose LD_{50} for T-2 toxin was 4·0 mg/kg body weight and for diacetoxyscirpenol, it was 5·0 mg/kg. T-2 toxin administered as 14 daily oral doses had an LD_{50} of 2·90 mg/kg, and diacetoxyscirpenol had an LD_{50} of 4·15 mg/kg. Combinations of the toxins caused additive lethal effects in both the single- and multiple-dose tests.

INTRODUCTION

The 12,13-epoxy-trichothecenes are a group of at least 40 structurally related toxic fungal metabolites, most of which are produced by Fusarium species (Mirocha, 1979). Four of these mycotoxins, T-2 toxin, diacetoxyscirpenol, deoxynivalenol and nivalenol, have been identified as toxic components of naturally contaminated feedstuffs. Of these four toxins, two have been found in a single feed sample on at least two occasions (Ghosal, Biswas, Srivastava, Chakrabarti & Basu Chaudhary, 1978; Mirocha, Schauerhamer, Christensen & Kommedahl, 1979), and several species of Fusarium can produce combinations of these and other trichothecenes when cultured under laboratory conditions (Ciegler, 1978; Ueno, Ishii, Sawano, Ohtsubo, Matsuda, Tanaka, Kurata & Ichinoe, 1977). Synergistic lethal toxicity of three metabolites of *Penicillium viridicatum* (ochratoxin A, citrinin, and penicillic acid) was demonstrated in female mice (Sansing, Lillehoj, Detroy & Miller, 1976).

This paper describes the toxicity of T-2 toxin and diacetoxyscirpenol, given separately and in combinations as single and multiple doses, in male broiler chickens.

EXPERIMENTAL

Chickens. Male broiler chicks of the Hubbard × Hubbard strain were obtained when one day old from a commercial hatchery (Fairview Farms, Remington, IN). The parent flock was seronegative to *Mycoplasma gallisepticum* and *M. synoviae*, and the chicks received Marek's disease vaccine prior to shipment. They were reared in wire-floored brooding cages measuring 60 × 90 × 20 cm, with a brooding temperature of 35–40°C, a room temperature of 22°C, continuous illumination, and forced air ventilation. A maximum of 50 birds were housed in each cage until they were 14 days old when the maximum density was reduced to 32 birds per cage. Non-medicated commercial starter mash for broiler chicks and fresh tap water were available *ad lib.* The chicks were acclimatized and observed daily but not handled until they were 7 days old, when the body weight was about 100 g. Sequentially numbered aluminium wingbands were randomized, affixed to the wing web, and the birds were placed in dose groups according to the wingband number sequence.

Toxins and solvent. T-2 toxin was prepared by B. Yagen and diacetoxyscirpenol was obtained from Makor Chemical, Ltd. (Jerusalem, Israel). Each toxin was dissolved in one part reagent-grade dimethylsulphoxide and then diluted to a final volume with nine parts of physiological saline to make stock solutions of appropriate concentrations for administration to the high-dose groups. These stock toxin solutions were diluted to the desired concentrations with a stock solvent solution of dimethylsulphoxide:saline (1:9 v/v). The solvent exposure was 10 ml/kg body weight regardless of the toxin dose. For the multiple dose study, the toxic solutions were prepared daily and administered within 3 hr of preparation.

Toxin administration. Single oral dose 72-hr LD_{50} determinations were made for each toxin given separately and in dosage combinations presented in Table 1. The toxins were administered into the crop using a 1-ml plastic tuberculin syringe and a curved 7·5-cm dosing needle. Feed was withheld 4 hr prior to dosing. The LD_{50} was calculated by the method of Litchfield & Wilcoxon (1949) and the joint potency was defined by the isobologram method of Hewlett (1969) for two drugs of separate activity. Ten birds were in each dose group including a solvent-only group.

Multiple oral dose LD_{50} determinations were based on 14 consecutive daily doses administered to dose groups described in Table 1. The method of

Table 1. *T-2 toxin and diacetoxyscirpenol combinations and LD_{50}s for 72-hr single and 14-day multiple oral toxicity tests*

T-2 toxin: diacetoxyscirpenol dosages (mg/kg body weight)	Mortality (no. of deaths/ no. in group)	LD_{50} for toxin combinations with 95% confidence intervals
	Single-dose test	
6·0:0	7/10	4·0:0
5·0:0	9/10	(2·74–5·84:0)
4·0:0	5/10	
3·0:0	0/10	
2·0:0	1/10	
1·0:0	0/10	
4·5:1·75	10/10	
4·0:1·5	9/10	3·25:1·14
3·5:1·25	5/10	(3·01–3·51:1·06–1·22)
3·0:1·0	4/10	
2·4:2·4	5/10	
2·2:2·2	4/10	2·26:2·26
2·0:2·0	4/10	(1·74–2·94:1·74–2·94)
1·8:1·8	1/10	
1·6:1·6	1/10	
1·75:4·5	10/10	
1·5:4·0	10/10	1·03:3·05
1·25:3·5	9/10	(0·82–1·28:2·44–3·81)
1·0:3·0	5/10	
0:6·0	4/10	
0:5·0	5/10	
0:4·0	5/10	0:5·0
0:3·0	0/10	(0:4·03–6·20)
0:2·0	0/10	
0:1·0	0/10	
	Multiple-dose test	
3·0:0	5/10	
2·5:0	4/10	2·90:0
2·0:0	0/10	(2·36–3·57:0)
1·5:0	0/10	
2·50:1·0	5/8	
2·25:0·75	3/8	2·38:0·88
2·00:0·50	1/8	(2·14–2·64:0·66–1·17)
1·75:0·25	0/8	
1·75:1·75	5/8	
1·50:1·50	3/8	1·55:1·55
1·25:1·25	2/8	(1·38–1·74:1·38–1·74)
1·00:1·00	0/8	
1·0:2·50	5/8	
0·75:2·25	0/8	0·88:2·38
0·50:2·00	2/8	(0·64–1·21:2·13–2·67)
0·25:1·75	0/8	
0:3·5	2/10	0:4·15
0:3·0	1/10	(0:3·29–5·23)
0:2·5	2/10	

toxin administration was similar to that in the single-dose study except that feed was withheld 2 to 3 hr prior to dose administration. For individual toxin administration and solvent-only controls, there were ten birds per dose group. Eight birds were in each group that received the combined toxins.

RESULTS

Single-dose toxicity

The toxins administered alone and in combinations usually caused death within 24 hr of dosing. Affected birds typically lost 10% of their body weight and had clear or white opaque liquid faecal droppings. Decreased spontaneous activity was followed by sternal recumbency and death. The single oral dose LD_{50} determinations are presented in Table 1. From these data plotted on an isobologram (Fig. 1), it was concluded that T-2 toxin and diacetoxyscirpenol have additive lethal toxicity when dosed in combination. The dashed line in Fig. 1 represents the line of additive action and the three combination LD_{50} values lie on either side of this line, a feature of additive action.

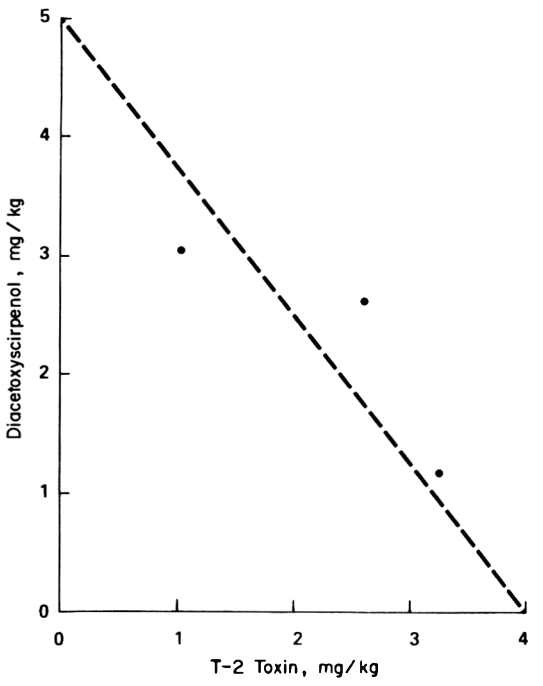

Fig. 1. Isobologram of the joint potency of T-2 toxin and diacetoxyscirpenol as defined by the single oral dose LD_{50}. The dashed line represents assumed additive toxicity.

Multiple-dose toxicity

Chicks that died of multiple doses had clinical signs similar to those described in the single-dose study. Most of the birds that died succumbed by the seventh day. The surviving chicks had decreases of body weight gain and packed cell volume. The LD_{50} values for the multiple dose study are presented in Table 1. Application of the isobologram method of analysis (Fig. 2) to these data indicates additive lethal toxicity for T-2 toxin and diacetoxyscirpenol when administered in combinations as 14 consecutive daily oral doses.

DISCUSSION

This study defined the lethal effects of two naturally occurring trichothecene mycotoxins, T-2 toxin and diacetoxyscirpenol, administered in combination. The results indicated additive toxicity and should be useful in the correlation of severity of clinical disease with data obtained from the analysis of toxic feedstuffs. There are several reasons, however, why the results should be interpreted within the limits of conditions of this study.

Only male chicks were used and sex has been shown to be an important variable when evaluating the interactions of mycotoxins. Thacker (1976) concluded that ochratoxin A and citrinin had synergistic lethal toxicity in female guinea-pigs but the effects were additive in males. This may be important for trichothecene mycotoxins because of their natural occurrence in association with zearalenone (Ghosal *et al.* 1978; Mirocha *et al.* 1979), a commonly encountered mycotoxin with oestrogenic biological activity.

Secondly, the interaction of T-2 toxin and diacetoxyscirpenol was defined only by the lethal toxicity. The sublethal toxicity of two mycotoxins in combination can be more complex. Sterigmatocystin and aflatoxin B_1 administered in combination to guinea-pigs caused a synergistic increase in the serum albumin, but decreases of α_2-globulin, β-globulin, and complement activity were additive effects (Richard, Thurston, Lillehoj, Cysewski & Booth, 1978). The contributions of these various factors could modify the severity of clinical disease.

A third reason involves understanding the role of mycotoxins and their contributions to disease in the presence of other important factors. Hamilton (1977) reviewed substantial data and concluded that mycotoxins can have synergistic detrimental effects in combination with nutritional deficiencies. The same may also hold true for infectious diseases, an area of special importance for the trichothecene mycotoxins because of their detrimental effects on the immune system (Boonchuvit, Hamilton & Burmeister, 1975). Thus, the effects of one or more mycotoxins in feedstuffs must be evaluated with regard to possible concurrent nutritional or infectious diseases.

T-2 toxin was more toxic than diacetoxyscirpenol in both the single- and multiple-dose tests conducted in this study. These results differ from previously reported data that showed diacetoxyscirpenol to be more toxic than T-2 toxin in chickens (Chi, Robison, Mirocha & Reddy, 1978). The two derivatives differ structurally only in that T-2 toxin possesses an isovaleroxy group at carbon-8 and diacetoxyscirpenol has an H_2 group in that position (Mirocha, 1979). While the differences in toxicity are small, they indicate that

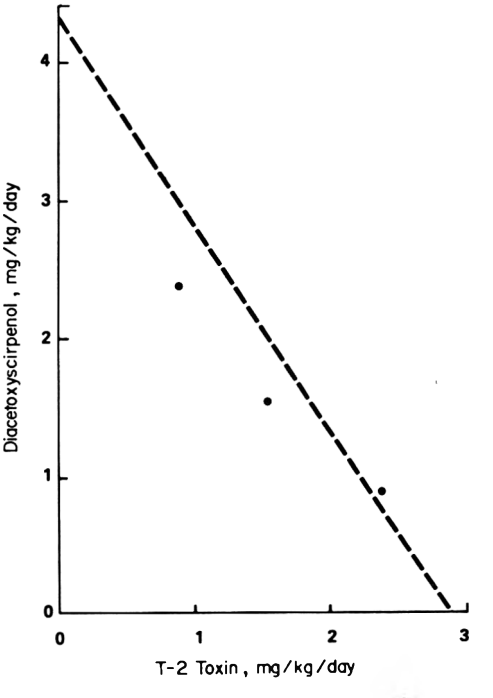

Fig. 2. Isobologram of the joint potency of T-2 toxin and diacetoxyscirpenol as defined by the 14-day multiple oral dose LD_{50}. The dashed line represents assumed additive toxicity.

the functional group at the C-8 position may have a role in the biotransformation of the trichothecenes in chickens, as was found in rabbits (Ohta, Matsumoto, Ishii & Ueno, 1978). The metabolic role of the specific groups positioned at C-8 could, however, be different for the two species. In rabbits, the C-8 moieties of T-2 toxin and diacetoxyscirpenol did not affect de-acetylation of the C-4 position by hepatic carboxylesterase, which is the major mechanism of biotransformation. This type of comparative study has not yet been published for chickens.

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EFFECT OF 2,4,5-TRICHLOROPHENOXYACETATE ON RENAL FUNCTION

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Abstract—2,4,5-Trichlorophenoxyacetate (2,4,5-T) has been shown to undergo renal tubular secretion in the isolated perfused rat kidney, especially when a dextran perfusate was used to limit 2,4,5-T–colloid binding. This renal transport of 2,4,5-T led to a high concentration of 2,4,5-T in the kidney and a resultant decrease in the functional capacity of the perfused kidney. The present study investigated the effect of 2,4,5-T on *in vivo* renal function in the rat. When 100 mg 2,4,5-T/kg was infused iv, no effect on renal function was observed; glomerular filtration rate (GFR), fractional reabsorption of water and electrolytes, (FR_{H_2O}, FR_{Na}, FR_K, FR_{Cl}), urine flow rate ($\dot{V}$) and clearance of tetraethylammonium (C_{TEA}) were unaltered. One possibility for the discrepancy between the findings in the perfused kidney and in the *in vivo* experiments was the high degree of plasma binding of 2,4,5-T found in rat plasma. This plasma-protein binding, which has been known to decrease 2,4,5-T excretion, appeared to protect the kidney from the compound's acute nephrotoxic effects. To test this hypothesis, perfused-kidney experiments were performed with 2,4,5-T in a bovine serum-albumin perfusate. Under these conditions, there was no change in the GFR, $\dot{V}$, FR_{Na}, FR_{H_2O} and FR_{Cl}. The clearance of *p*-aminohippurate (C_{PAH}), however, was reduced in the presence of 2,4,5-T. The C_{PAH}/GFR was decreased from about 6 to 1, which indicated that organic-anion transport was completely inhibited by a 2,4,5-T concentration that was not nephrotoxic. Thus 2,4,5-T did not produce acute renal failure *in vivo* but was still able to cause a selective inhibition of organic-anion transport in the kidney.

INTRODUCTION

Numerous studies have demonstrated that the rat kidney transports certain organic anions by an active carrier mechanism which has been located in the proximal tubular cell. Two organic anions, penicillin and *p*-aminohippurate (PAH), have been shown to undergo renal proximal tubular secretion by a highly specific mechanism (Weiner, 1973). The rat kidney has also been shown to secrete 2,4,5-trichlorophenoxyacetate (2,4,5-T; auxin-type herbicide) by the PAH transport mechanism (Berndt & Koschier, 1973; Hook, Bailie, Johnson & Gehring, 1974; Koschier & Acara, 1979). However, experiments with the isolated perfused kidney (Koschier & Acara, 1979) indicated that the renal clearance of 2,4,5-T was less than that of PAH. One contributing factor was that 2,4,5-T was found to be extensively bound to plasma proteins and this binding limited the amount of 2,4,5-T available for secretion. Nevertheless, 2,4,5-T underwent net tubular secretion even in a solution in which approximately 99% of the herbicide was bound to plasma protein.

A high concentration of 2,4,5-T has been found to be nephrotoxic to the perfused kidney. When a rat kidney was perfused with a dextran medium containing 1 mM-2,4,5-T, the glomerular filtration rate, fractional reabsorption of sodium, urine flow rate, clearance of tetraethylammonium and clearance of PAH were decreased markedly (Koschier & Acara, 1979). The initial perfusate concentration in these studies was similar to plasma levels (2–3 mM) found in studies in which rats given 100 mg 2,4,5-T/kg iv showed a decreased ability to excrete 2,4,5-T in the urine compared with those given 5 mg/kg (Sauerhoff, Braun, Blau & Gehring, 1976).

The present investigation measured renal function in rats given 100 mg 2,4,5-T/kg to determine whether this herbicide could produce alterations in renal function *in vivo*. The rationale for this study was based on the fact that 2,4,5-T was shown to be transported and accumulated by the kidney during *in vitro* and *in vivo* experiments (Koschier & Acara, 1979; Piper, Rose, Leng & Gehring, 1973; Sauerhoff *et al.* 1976). The resulting concentration of 2,4,5-T in the kidney could alter renal function, as has been demonstrated for other compounds (*bis*-(*p*-chlorophenyl) acetic acid for example; DDA) even when they were known to be extensively bound to plasma proteins (Koschier & Hong, 1980).

EXPERIMENTAL

Test material. Analytical-grade 2,4,5-trichlorophenoxyacetic acid (AGR 86187) was supplied most generously by Dow Chemical Co., Midland, MI. The compound was first converted to the sodium salt by the addition of a slight excess of NaOH and then brought to pH 7·40. Since this compound has a pKa of approximately 3, the herbicide exists as an organic anion at physiological pH.

In vivo *renal clearance experiments.* The methodology used was modelled on the renal clearance protocol of Bowman, Arnow & Weiner (1978). Male

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Sprague–Dawley rats (350–450 g) were anaesthetized with an ip injection of 100 mg Inactin/kg (Promonta Corp., Hamburg, FRG). A tracheostomy was performed and a polyethylene tube (PE240; Clay Adams, Parsippany, NJ) was inserted into the trachea to ensure a clear airway. The urinary bladder was exposed through a midline incision, and a catheter (PE50; Clay Adams) was inserted. The left external jugular vein was cannulated with a catheter for infusion of solutions (Tygon surgical tubing, 0·025 in. ID and 0·04 in. OD, from Norton Plastics, Akron, OH). The same type of catheter was inserted into the right carotid artery for obtaining blood samples and for monitoring blood pressure (Statham Instruments, Oxnard, CA; Grass Instruments, Quincy, MA). The temperature of the rat was continuously monitored by means of a rectal thermistor probe connected to a telethermometer (Yellow Springs Instruments, Yellow Springs, OH). Body temperature was maintained between 36 and 38°C, using the heat from a lamp.

Throughout a 150-min infusion, four control and three treated rats were infused at a rate of 0·193 ml/min with isotonic saline containing 5% mannitol, 10 mM-KCl, 11·1 μM-[^{14}C]TEA and 0·332% [^{3}H]polyethylene glycol ([^{3}H]PEG; mean mol wt 4000). For the first 90 min, the infusate given to the test rats also contained 2,4,5-T in a concentration to provide a dose of 100 mg/kg during this period, after which the control solution containing no 2,4,5-T was infused. The first 30 min of infusion was considered an equilibration period, at the end of which urine collections were started and continued for 120 min. Urine from each animal was collected for six consecutive 20-min periods and blood samples (approximately 0·6 ml) were taken at the mid-point of each of these periods. The volume of each blood sample was replaced immediately with resuspended blood cells from the previous collection period. The resuspension contained 20 U heparin/ml.

For each collection period, the renal function of the rats was assessed in terms of the urinary flow rate ($\dot{V}$), the plasma clearance of PEG as a measure of the glomerular filtration rate (GFR), the plasma clearance of TEA (C_{TEA}) as an index of renal plasma flow, and the mean arterial blood pressure (MABP). The fractional reabsorption of sodium, chloride and potassium ions, and of water (FR_{Na}, FR_{Cl}, FR_K and FR_{H_2O}) was also determined.

Perfused kidney experiments. The isolated perfused kidney experiments were performed with male Sprague–Dawley rats, according to the method outlined by Bowman (1975) with the major modifications described by Koschier & Acara (1979). The basic composition of the perfusate was as follows: 6% (w/v) fraction V bovine serum albumin (BSA); 143 mM-Na^+; 5·9 mM-K^+; 2·5 mM-Ca^{2+}; 1·2 mM-Mg^{2+}; 116·7 mM-Cl^-; 25 mM-HCO_3^-; 1·2 mM-SO_4^{2-}; 1·2 mM-$H_2PO_4^-$; 5·5 mM-glucose; 10 μm [^{14}C]PAH and 2 mg [^{3}H]PEG/ml. The perfusate was gassed with O_2–CO_2, 95:5 (v/v) and was adjusted to pH 7·4 at 37°C. 2,4,5-T was added to this perfusate in a concentration of 2 mM. The initial perfusate volume in the recirculating system was 75 ml and, at the start of perfusion, fresh perfusate was pumped into the recirculating system at a rate of 0·097 ml/min. The perfusion was continued for 120 min, during which urine was collected for each 10 min period and perfusate samples (1 ml) were taken at the midpoint of each urine collection.

Binding of 2,4,5-T to plasma proteins. To determine the amount of 2,4,5-T that was bound to BSA in the perfusate, ultrafiltration of the perfusates was performed, using the method described by Chignell (1977). Specifically, Spectrapor 2 dialysis membranes (Spectrum Medical Industries, Los Angeles, CA) were filled with 2 ml perfusate or perfusate without BSA. The membranes were spun for 40 min (22°C) at 300 **g**, which filtered about 5–10% of the perfusate, and the filtrate was removed. The perfusates were respun and the calculated amount of 2,4,5-T binding to the perfusate for the second run was recorded.

Analytical methods. Urine samples were collected in preweighed vials and the urine volume was determined gravimetrically by assuming a urine specific gravity of 1·0. The amounts of radiolabelled compounds in the samples of urine, plasma and perfusate were determined in a Beckman 350 liquid scintillation counter, and Aquasol (New England Nuclear, Boston, MA) was used as the scintillation counting solution. For all counting conditions, the degree of quench was determined by the use of standard techniques previously described (Koschier, Conway & Rennick, 1979). A Radiometer FLM3 (Radiometer, Copenhagen, Denmark) flame photometer was used to determine the concentration of Na and K in samples. The chloride concentration of samples was measured with a Radiometer CMT 10 chloride titrator (Radiometer, Copenhagen).

The data were analysed statistically using Student's *t* test or a paired *t* test. The level of probability taken to denote significance was $P < 0·05$.

RESULTS

The effect of 2,4,5-T on renal function *in vivo* has been detailed in Fig. 1. There was no significant difference between the renal function of the control and 2,4,5-T groups when each time period was compared. The parameters $\dot{V}$, GFR, C_{TEA}, C_{TEA}/GFR and MABP were all unaffected by the administration of 2,4,5-T (Fig. 1a), as were the FR_{H_2O}, FR_{Na}, FR_{Cl} and FR_K (Fig. 1b). The lack of any effect on *in vivo* renal function was in sharp contrast to the effects of this herbicide on renal function in the isolated perfused kidney (Koschier & Acara, 1979).

In the isolated perfused kidney experiments performed to test the possibility that plasma binding of 2,4,5-T would limit the acute nephrotoxicity of the herbicide, about 97% of the 2,4,5-T present at a level of 2 mM in perfusate containing 6% BSA was found to be bound to the protein (Table 1). This 2 mM concentration of 2,4,5-T was approximately the same as the plasma concentration of 2,4,5-T after administration of an iv dose of 100 mg/kg (Sauerhoff *et al.* 1976). There was no significant difference between renal function in the control and in the 2,4,5-T kidneys when each time period was compared (Fig. 2); $\dot{V}$, GFR, FR_{H_2O}, FR_{Na} and FR_{Cl} were unaffected by 2,4,5-T during the 120-min renal perfusion. However, the renal tubular secretion of PAH was almost completely inhibited by the presence of 2,4,5-T (Fig. 3). After an initial equilibration period, the C_{PAH}/GFR averaged 5–7 in the control experiments. When

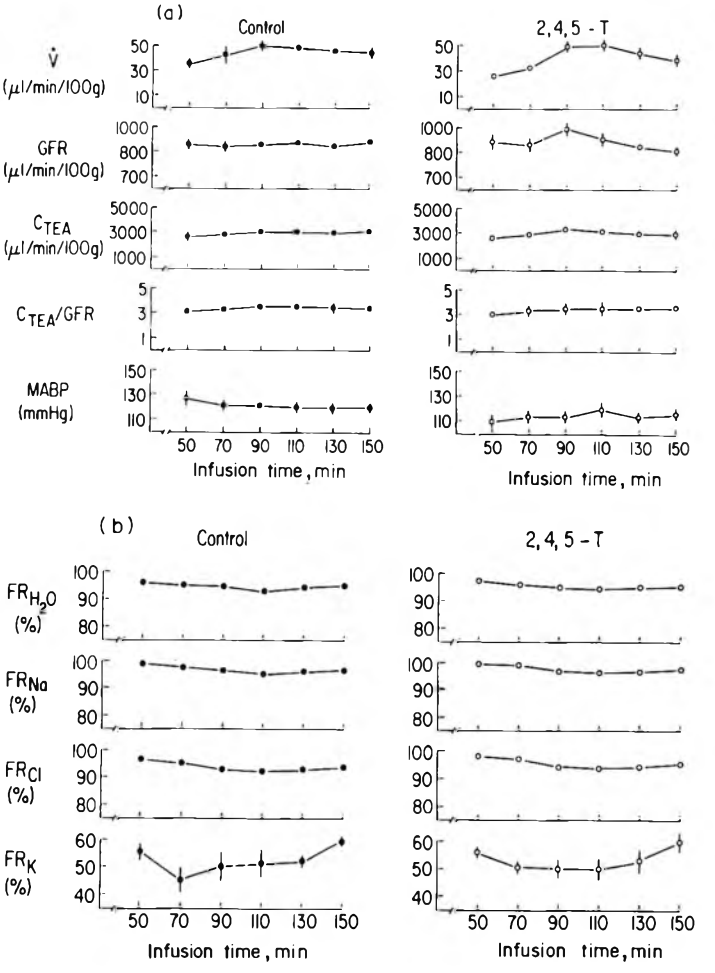

Fig. 1. The effect of 2,4,5-T on (a) renal function and (b) reabsorption of inorganic ions and water in three rats (○) infused over 90 min with 100 mg 2,4,5-T/kg in saline followed for 60 min with the control saline infusion, which was similarly administered to a group of four control rats (●) throughout the 150-min experiment. For each group the first 30-min was considered an equilibration period, which was followed by six 20-min collection periods. For each period, the following indicators of renal function were measured: urinary flow rate (V), glomerular filtration rate (GFR), clearance of tetraethylammonium (TEA) and mean arterial blood pressure (MABP). Fractional reabsorption (FR) was recorded for water and for sodium, chloride and potassium ions.

2,4,5-T (2 mM) was present in the perfusate, the C_{PAH}/GFR decreased to approximately 1. In the control experiments, the perfusate concentration of PAH remained constant at about 0·4 μM after the first 40 min of perfusion. When 2,4,5-T was present in the perfusate, the removal of PAH from the perfusate was much lower and the plasma level was approximately 4·0 μM after 60 min of perfusion.

Table 1. *Binding of 2,4,5-T in isolated-kidney perfusate containing 2 mM 2,4,5-T with or without 6% BSA*

Perfusate	2,4,5-T bound (%)*
BSA (%)	98·4 ± 0·16
Buffer without colloid	1·04 ± 1·66

BSA = Bovine serum albumin
*Values are means ± SEM; samples were assayed in triplicate.

DISCUSSION

The results from this study indicated that the iv administration of 2,4,5-T in a dose of 100 mg/kg did not produce any acute alterations in the renal function of the rat. Certain renal tubular transport processes and the blood flow to the kidney were apparently also unaffected by 2,4,5-T. This response differed from the effect of 2,4,5-T demonstrated in an earlier study in the isolated perfused kidney, in which 1 mM-2,4,5-T in a dextran perfusate had a prompt and significant effect on the GFR, FR_{Na}, V̇, C_{TEA} and C_{PAH} (Koschier & Acara, 1979).

The apparent cause for the discrepancy between that perfused-kidney study and the *in vivo* clearance experiments reported here was the high degree of plasma binding of 2,4,5-T in rat plasma. The plasma-protein binding of 2,4,5-T appeared to have protected the kidney from the acute nephrotoxic effects of the herbicide. Since plasma-protein binding of many compounds has been widely known to limit their

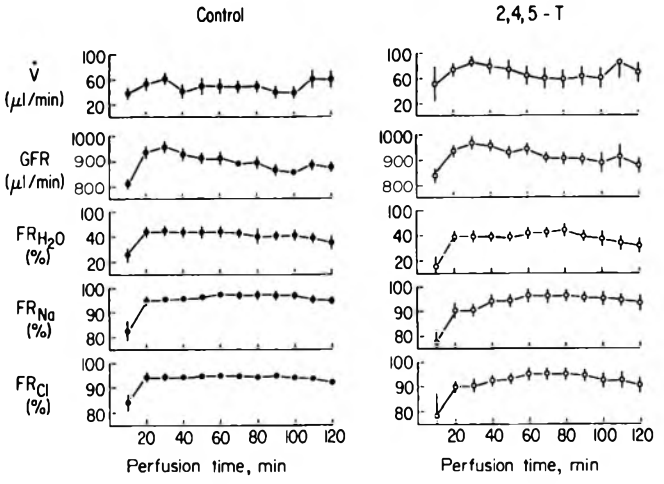

Fig. 2. The effect of 2 mM-2,4,5-T on the function of isolated kidneys perfused with a medium containing 6% BSA. Renal function was determined for each successive 10 min from the start of perfusion, on the basis of the urinary flow rate (V̇), glomerular filtration rate (GFR) and fractional reabsorption (FR) of water, sodium and chloride. Values are means for 3 kidneys perfused with 2,4,5-T (○) compared with those for five control perfusions (●).

tissue accumulation, this finding could be viewed as unremarkable and predictable. On the other hand, numerous studies have shown that renal secretion of some organic ions occurred even when they were bound to plasma proteins (Weiner, 1973). Experiments have demonstrated proximal tubular secretion even when 2,4,5-T was approximately 99·5% bound to plasma protein (Koschier & Acara, 1979). In addition, the organic anion DDA has been shown to be extensively bound to plasma proteins (*c.* 97%) and still to be able to produce dramatic alterations in renal functions, the GFR, V̇ and FR$_{Na}$ being decreased significantly. Therefore, the relationship between the plasma binding of 2,4,5-T and its degree of nephrotoxicity was not obvious and needed detailed investigation.

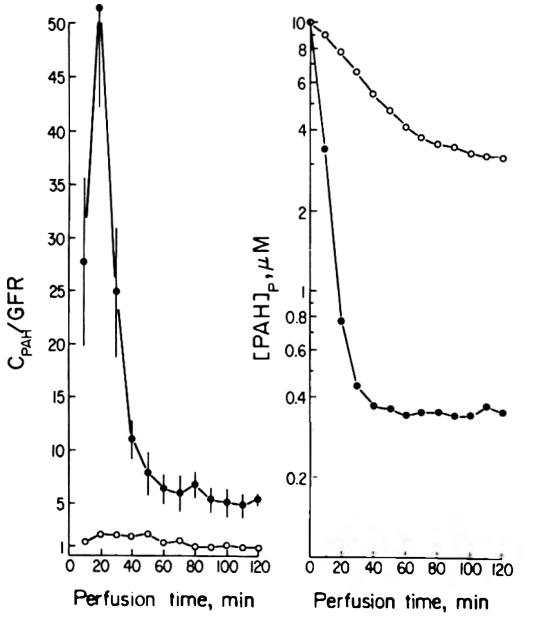

Fig. 3. The effect of 2,4,5-T on the renal secretion of PAH and on the concentration of PAH in the perfusate from the isolated kidneys perfused with 2,4,5-T (○) or the control perfusate (●) as described for Fig. 2.

Even though 2,4,5-T did not alter certain renal functions, the C_{PAH}/GFR was decreased from about 6 to 1 by the addition of 2,4,5-T to the perfusate. This indicated that organic-anion transport could be completely inhibited by a concentration of 2,4,5-T that was not nephrotoxic and suggested that in the presence of 2,4,5-T renal excretion rates would be reduced for the numerous xenobiotics secreted by the proximal tubule, such as saccharin, citrinin, morphine ethereal sulphate, penicillin, salicylate, salicylglucuronides and 2,4-dichlorophenoxyacetate (Berndt & Koschier, 1973; Goldstein, Hook & Bond, 1978; Phillips, Berndt & Hayes, 1979; Weiner, 1973).

Since 2,4,5-T blocked the renal tubular secretion of PAH, it was apparent that even though 2,4,5-T was largely bound to plasma protein, some was available for secretion, as was previously noted by Koschier & Acara (1979). The proximal tubular transport of 2,4,5-T has been shown to concentrate this herbicide in the kidney, an effect that is possibly augmented by the intracellular binding of 2,4,5-T (Koschier, Hong & Berndt, 1979b). In view of the ability of the kidney to transport and bind 2,4,5-T, this herbicide could accumulate to a high level over a period of time, after which alterations in renal function could be produced. High tissue levels have been produced in recent experiments (Piper *et al.* 1973; Sauerhoff *et al.* 1976); after 2,4,5-T was administered iv at a dose of 100 mg/kg, the plasma level was *c.* 0·5–2 mM and the kidney concentration was 0·2–0·3 mg/g. These levels persisted for up to 32 hr and then decreased only slowly. While high doses of 2,4,5-T could have some nephrotoxic potential, lower doses have been shown to be excreted rapidly by the kidney and no alterations in renal function have been found after repeated daily doses of 2,4,5-T (Koschier & Berndt, 1977; Piper *et al.* 1973).

In conclusion, the results indicated that 2,4,5-T did not produce acute renal failure under the *in vivo* conditions of this study. However, this herbicide was still

able to exert a selective inhibition on organic-anion transport in the kidney even when it was bound extensively to plasma protein.

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PERCUTANEOUS PENETRATION OF MOSQUITO REPELLENTS IN THE HAIRLESS DOG: EFFECT OF DOSE ON PERCENTAGE PENETRATION*

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Abstract—The percentage penetration of *N,N*-diethyl-*m*-toluamide (*m*-deet) and 2-ethyl-1,3-hexanediol (ethylhexanediol) was determined on hairless dogs at a dose of 4 $\mu g/cm^2$, and these results were compared with corresponding data at 320 $\mu g/cm^2$. In addition, the percentage percutaneous penetration for *n*-butanesulphonamide cyclohexamethylene (sulphonamide), an experimental mosquito repellent, was determined at doses of 100, 320 and 1000 $\mu g/cm^2$ in the hairless dog. The mean percentage penetration was found to decrease with increasing chemical dose for sulphonamide and *m*-deet, and to increase slightly with ethylhexanediol, but none of these changes differed significantly at the 95% confidence level.

INTRODUCTION

Many studies of percutaneous penetration are conducted using a single dose of the chemical per unit skin area. This dose may or may not correspond to estimates of actual human exposure to the chemical. In studies involving both man and rhesus monkey, Wester & Maibach (1976) have shown that the percutaneous penetration (expressed as a percentage of the applied dose) of testosterone, hydrocortisone and benzoic acid increases as the topical dose decreases. Using an *in vitro* apparatus with human skin, Scheuplein & Ross (1974) also reported a decrease in percentage percutaneous penetration with increase in chemical dose for cortisone. As expected, the total amount of the compound absorbed increased as the dose increased in these studies. If percentage percutaneous penetration is dependent on the dose applied, permeability experiments for estimating penetration in man should be carried out either with a test dose corresponding to the likely level of human exposure or with more than one dose.

In the conduct of our efficacy tests of repellents against mosquitoes, our standard chemical dosage has been 320 $\mu g/cm^2$, applied to the skin of volunteers or to our hairless dog animal model (Hill, Robinson, McVey, Akers & Reifenrath, 1979). This dose is comparable to the amount an individual would apply personally to his or her forearms (W. G. Reifenrath, unpublished data 1979). Accordingly, we have reported the percutaneous penetration of the most commonly used mosquito repellents, *N,N*-diethyl-*m*-toluamide (*m*-deet), its isomer *N,N*-diethyl-*p*-toluamide (*p*-deet) and 2-ethyl-1,3-hexanediol (ethylhexanediol), at a dose of 320 $\mu g/cm^2$ on the hairless dog (Reifenrath, Hill, Robinson, McVey, Akers, Anjo & Maibach, 1980). However, other published studies of the percutaneous penetration of the mosquito repellents *m*-deet (Feldmann & Maibach, 1970) and *n*-butanesulphonamide cyclohexamethylene ('sulphonamide'; Snodgrass & Weeks, 1978) used a dose of 4 $\mu g/cm^2$. This dose is below the minimum effective dose for both *m*-deet and sulphonamide against *Aedes aegypti* mosquitoes (Hill *et al.* 1979). If percutaneous penetration (expressed as a percentage of the applied dose) increases with a decrease in the applied dose of repellent, the percentage penetration derived at 4 $\mu g/cm^2$ may be unrealistically high.

To investigate the influence of the dose of the chemical on the percentage percutaneous penetration of repellents, we used the hairless dog, the same animal model we had found suitable in previous studies (Reifenrath *et al.* 1980). Now we report the percutaneous penetration of *m*-deet and ethylhexanediol in the hairless dog at a dose of 4 $\mu g/cm^2$ for comparison with previously reported data 320 $\mu g/cm^2$ (Reifenrath *et al.* 1980) as well as the penetration of the experimental repellent sulphonamide at doses of 100, 320 and 1000 $\mu g/cm^2$ in the hairless dog.

EXPERIMENTAL

Test materials. The following mosquito repellents were used: *N,N*-diethyl-*m*-toluamide (*m*-deet) from Eastman Chemical Co., Rochester, NY, 2-ethyl-1,3-hexanediol (ethylhexanediol) from Niagara Chemical Division, FMC, Middleport, NY, and *n*-butanesulphonamide cyclohexamethylene ('sulphonamide') reported by Pervomaiskii, Osipyan, Kazhdan, Maslii, Shustrov, Grabovskii, Zhogolov & Dunavea (1967). Their radiolabelled counterparts were: [^{14}C-carbonyl]-*N,N*-diethyl-*m*-toluamide, radiochemical purity 92% (Kurtz 1971); [1,3-^{14}C]-2-ethyl-1,3-hexanediol, radiochemical purity 99%, New England Nuclear, Boston, MA; and *n*-butanesulphonamide-[2,2′-^{14}C]-cyclohexamethylene, radiochemical purity 90%, SRI International, Menlo Park, CA.

*The opinions or assertions herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

Animals. Eighteen adult hairless dogs of either sex were selected from a colony of healthy dogs maintained at Letterman Army Institute of Research. Their size and weight varied from 11 to 23 kg. The same animals were used to compare percutaneous penetration of a given compound at different doses. Prior to repeated use, dogs were rested for at least 3 wk after the urine level of radioactivity had fallen to background. In conducting this research, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

Test procedure. The experimental procedure of Reifenrath *et al.* (1980) was used with some modifications. A minimum radioactive dose of 0·5 μCi was used in all trials. The low specific activity of the radiolabelled sulphonamide (332 dpm/μg) limited the lowest topical dose to 100 μg/cm^2; this was compared to higher topical doses of 320 and 1000 μg/cm^2 and required larger application sites (one 50-cm^2 site for the 320- and 1000-μg/cm^2 doses and two 50-cm^2 application sites, one on each side of the dog, for the 100-μg/cm^2 dose). The protective foam pad, similar to that previously used, had an 8 × 10·3-cm area cut out to accommodate the larger application area of 6 × 8·3 cm. To estimate the efficiency of urinary and faecal excretion of sulphonamide following iv administration, the low specific activity required a chemical dose of 5 mg/dog. For *m*-deet and ethylhexanediol, the higher specific activities ($1{\cdot}15 \times 10^4$ and $6{\cdot}06 \times 10^4$ dpm/μg, respectively) of the radioactive samples allowed comparison of a topical dose of 4 μg/cm^2 with the previously reported 320-μg/cm^2 dose. The specific activities of the radiolabelled samples were adjusted, when appropriate, with cold chemical to give the radioactive doses indicated in the tables.

Radioactivity determinations. Radioactivity in urine, protective patches and skin washings was determined as described by Reifenrath *et al.* (1980). Each day's collections of faeces were freeze-dried (Model 50 SRC Virtis Sublimator, Virtis Co., Gardiner, NY), and the samples were than placed into zip-lock polyethylene bags and ground to a fine powder. A 0·2-g aliquot was taken from each sample, mixed with 0·2 g cellulose powder and oxidized in a Packard Model 306 Sample Oxidizer (Packard Instruments, Des Plaines, IL) in which the radioactivity was converted to $^{14}CO_2$ and trapped in Packard Carbo-sorb. The radioactivity in the vials was counted on a Packard Model 3375 scintillation counter, spiked, and recounted to determine the disintegrations/min.

For determining radioactivity in blood samples, a 0·5-g aliquot of whole blood was placed in a Packard Combusto-cone. Following addition of 200 μl Packard Combust-aid to the sample, a Packard Combusto-Pad was placed over the solution, and the sample was oxidized in the Sample Oxidizer. The radioactivity in the vials was counted in the same manner as for faeces to determine the aliquot disintegrations/min.

RESULTS

The disposition of radioactivity following topical application of radiolabelled *m*-deet at 4 and 320 μg/cm^2 and ethylhexanediol at 4 μg/cm^2 is reported in Table 1, along with summary data for ethylhexanediol at 320 μg/cm^2. Urinary excretion of radioactivity *v.* time following treatment with these compounds is given in Table 2. The disposition of radioactivity following administration of radiolabelled sulphonamide topically at 100, 320 and 1000 μg/cm^2 and iv at 5 mg/dog is given in Table 3 and the time course of urinary excretion of radioactivity by these animals is given in Table 4. Table 5

Table 1. *Disposition of radioactivity following topical application of radiolabelled* m-*deet or ethylhexanediol to hairless dogs*

Dose (μg/cm^2)*	Dog no.	Recovery of radioactivity (% of applied dose)				
		Urine	Faeces	Skin†	Patch	Total
			***m*-Deet**			
4	2	9·9	NA	4·3	73·4	88·5
	8	16·4	NA	2·2	70·0	88·6
	9	8·5	NA	3·0	73·6	85·1
	Mean ± 1 SD...	11·6 ± 4·2	—	3·2 ± 1·1	72·6 ± 2·3	87·4 ± 2·0
320	2	11·3	0·6	0·9	70·8	83·6
	8‡	9·3	0·0	0·2	70·0	79·5
	9‡	4·8	0·0	0·1	77·9	82·8
	Mean ± 1 SD...	8·5 ± 3·3	0·2 ± 0·3	0·4 ± 0·4	72·9 ± 4·4	81·8 ± 2·0
			Ethylhexanediol			
4	1	9·1	NA	3·9	66·4	79·4
	4	5·8	NA	13·6	50·5	69·9
	6	7·9	NA	3·8	51·0	62·7
	Mean ± 1 SD...	7·6 ± 1·7	NA	7·1 ± 5·6	56·0 ± 9·0	70·7 ± 8·4
320‡	Mean ± 1 SD...	8·9 ± 1·6	0·0	0·7 ± 0·3	51·8 ± 11·5	61·8 ± 10·6

NA = Not assayed

*Approximate specific activities of these doses were 0·5 and 4 μCi for 4 and 320 μg *m*-deet/cm^2, respectively, and 0·5 and 5 μCi for 4 and 320 μg ethylhexanediol/cm^2, respectively.

†Skin was scrubbed with an alcohol-soaked cotton ball 48 hr after application.

‡Data from Reifenrath *et al.* (1980).

Table 2. *Urinary excretion of radioactivity* v. *time after topical administration of radiolabelled* m-*deet or ethylhexanediol to hairless dogs*

Dose ($\mu g/cm^2$)	Dog no.	Radioactivity (% of dose) in urine collected at (hr after application): 4	8	12	24	48	72	96	Total urinary radioactivity (% of dose)
					***m*-Deet**				
4	2	NU	NU	NU	7·9	0·8	0·9	0·3	9·9
	8	NU	NU	6·2	3·3	4·4	2·6	CT	16·4
	9	0·2	NU	2·7	2·0	2·2	1·4	CT	8·5
	Mean...	0·2	—	4·5	4·4	2·5	1·6	0·3	11·6 ± 4·2*
320	2	NU	NU	4·0	5·1	1·7	0·4	0·2	11·3
	8†	NU	NU	5·1	2·4	1·5	0·4	0·1	9·3
	9†	NU	0·7	0·8	0·9	2·0	0·2	0·2	4·8
	Mean...	—	0·7	3·3	2·8	1·6	0·3	0·2	8·5 ± 3·3*
					Ethylhexanediol				
4	1	NU	NU	2·5	2·9	2·6	0·8	0·3	9·1
	4	NU	NU	NU	2·7	1·4	1·4	0·3	5·8
	6	NU	NU	3·4	0·8	2·6	0·8	0·3	7·9
	Mean...	—	—	3·0	2·1	2·2	1·0	0·3	7·6 ± 1·7*

NU = No urination during collection period CT = Collections terminated

*Mean ± 1 SD.

†Data from Reifenrath *et al.* (1980).

presents blood levels of radioactivity following iv administration of sulphonamide and Table 6 the calculated percutaneous penetration of various doses of these repellents, expressed as a percentage of the applied dose and corrected for incompleteness of urinary excretion. Table 6 also shows the total amount absorbed per unit of skin area.

Most of the iv-injected radioactive dose of sulphonamide was recovered in the urine in 24 hr (Table 4); low levels of radioactivity were detected in the faeces (Table 3). Sulphonamide rapidly disappears from the systemic circulation, as evidenced by the low levels of radioactivity in the blood samples (Table 5). Our excretion data do not suggest that sulphonamide is temporarily pooled in the hairless dog, although a previous report indicated that this may have occurred in rabbits (Snodgrass & Weeks, 1978).

Following topical application of all the compounds, most of the urinary excretion of radioactivity occurred during the first 2 days (Tables 2 & 4). Only

Table 3. *Disposition of radioactivity following administration of radiolabelled* n-*butanesulphonamide cyclohexamethylene ('sulphonamide') topically at 100–1000 $\mu g/cm^2$ or by iv injection at a dose of 5 mg/dog to hairless dogs*

Dose*	Dog no.	Recovery of radioactivity (% of applied dose) Urine	Faeces	Skin†	Patch	Total
Topical ($\mu g/cm^2$):						
100	9	6·4	Tr	3·9	64·2	74·6
	10	10·2	Tr	4·0	56·0	70·3
	11	4·7	Tr	4·3	83·2	92·3
	Mean ± 1 SD...	7·1 ± 2·8	—	4·1 ± 0·2	67·8 ± 14·0	79·1 ± 11·7
320	9	6·0	Tr	2·7	84·6	93·3
	10	7·7	Tr	3·0	81·3	92·0
	11	4·1	Tr	4·2	81·6	89·9
	Mean ± 1 SD...	5·9 ± 1·8	—	3·3 ± 0·8	82·5 ± 1·8	91·7 ± 1·7
1000	9	3·2	Tr	1·5	94·0	98·8
	10	6·1	Tr	1·5	84·9	92·6
	11	3·3	Tr	1·4	90·1	94·9
	Mean ± 1 SD...	4·2 ± 1·7	—	1·5 ± 0·1	89·7 ± 4·6	95·4 ± 3·1
Intravenous:						
5 mg/dog	12	79·3	4·0	—	—	83·3
	13	76·5	0·0	—	—	76·4
	14	79·2	3·3	—	—	82·4
	Mean ± 1 SD...	78·3 ± 1·6	2·4 ± 2·1	—	—	80·7 ± 3·8

Tr = Traces of radioactivity (1% or less)

*Specific activities of these doses were 1·5, 1·6 and 1·2 μCi for the 100-, 320- and 1000-$\mu g/cm^2$ topical doses, respectively, and 0·5 μCi for the iv dose (5 mg/dog).

†Skin was scrubbed with an alcohol-soaked cotton ball 48 hr after application.

Table 4. *Urinary excretion of radioactivity v. time after topical or iv administration of radiolabelled* n-*butanesulphonamide cyclohexamethylene ('sulphonamide') to hairless dogs*

Dose	Dog no.	Radioactivity (% of given radioactive dose) in urine collected at (hr after application): 4	8	12	24	48	72	96	120	Total urinary radioactivity (% of dose)
Topical ($\mu g/cm^2$):										
100	9	0·1	1·1	0·9	1·4	2·7	0·2	0·1	CT	6·4
	10	NU	NU	3·8	3·6	2·4	0·4	0·1	CT	10·2
	11	NU	NU	1·8	1·1	1·6	0·2	0·1	CT	4·7
	Mean...	0·1	1·1	2·2	2·0	2·2	0·3	0·1	—	7·1 ± 2·8*
320	9	0·2	1·0	1·4	1·5	1·4	0·5	CT	CT	6·0
	10	NU	0·0	NU	5·5	1·7	0·4	CT	CT	7·7
	11	NU	NU	1·1	1·0	1·7	0·3	CT	CT	4·1
	Mean...	0·2	0·5	1·3	2·7	1·6	0·4	—	—	5·9 ± 1·8*
1000	9	NU	0·4	0·5	0·7	0·9	0·5	0·2	0·0	3·2
	10	NU	0·0	NU	3·7	1·6	0·7	0·1	0·0	6·1
	11	NU	0·0	0·3	0·4	1·7	0·4	0·1	0·4	3·3
	Mean...	—	0·1	0·4	1·6	1·4	0·5	0·1	0·1	4·2 ± 1·7*
Intravenous:										
5 mg/dog	12	NU	NU	63·4	NU	14·7	0·9	0·3	CT	79·3
	13	12·6	NU	NU	55·9	7·4	0·4	0·2	CT	76·5
	14	NU	NU	NU	73·7	4·8	0·6	0·1	CT	79·2
	Mean...	12·6	—	63·4	64·8	8·9	0·6	0·2	—	78·3 ± 1·6*

NU = No urination during collection period CT = Collections terminated

*Mean ± 1 SD.

traces of radioactivity (1% or less) could be detected in the faeces following topical application of sulphonamide at the various dosages. Faeces were not analysed in the trials of *m*-deet and ethylhexanediol at 4 $\mu g/cm^2$ since little or no radioactivity could be detected in faeces in trials at 320 $\mu g/cm^2$. Most of the topically applied radioactivity was recovered in the protective patch (Tables 1 and 3). As the patch did not touch the application site, the radioactivity it contained probably resulted from trapped evaporation

Table 5. *Blood levels of radioactivity following iv administration of radiolabelled* n-*butanesulphonamide cyclohexamethylene ('sulphonamide') at a dose of 5 mg/animal to hairless dogs*

Time after injection	Blood radioactivity (dpm/g whole blood) in dog no. 12	13	14	Mean radioactivity (dpm/g whole blood)
5 min	186	72	78	112
15	152	86	114	117
30	168	98	138	135
1 hr	140	72	94	102
2	84	38	60	61
4	38	22	16	25
8	28	6	14	16
24	10	2	4	5

Table 6. *Percutaneous penetration and total absorption of repellents in relation to the dose of the chemical applied to the hairless dog*

Compound	Topical dose ($\mu g/cm^2$)	Penetration (% of applied dose*)	Mean total absorbed ($\mu g/cm^2$)
Ethylhexanediol	4	8·8 ± 2·0	0·35
	320	10·3 ± 1·9	33·0
m-Deet	4	12·8 ± 4·6	0·51
	320	9·4 ± 3·6	30·1
Sulphonamide†	100	9·1 ± 3·6	9·1
	320	7·5 ± 2·2	24·0
	1000	5·4 ± 2·2	54·0

*Values (means ± 1 SD) are corrected for incompleteness of urinary excretion.
†*n*-Butanesulphonamide cyclohexamethylene.

and shed stratum corneum. The relative volatilities for the three repellents are: sulphonamide < *m*-deet < ethylhexanediol (J. Jaeger, Letterman Army Institute of Research, personal communication, June 1979). The overall recovery from the topical trials generally decreased with the more volatile compounds. This was to be expected, as the protective patch was designed to be non-occlusive.

Small amounts of radioactivity were recovered by scrubbing the skin surface 48 hr after application of the repellent (Tables 1 and 3). With all the compounds tested, a larger amount of repellent per unit area remained on the skin surface with increasing dose per unit area; however, a higher percentage of the applied dose was recovered from the lower topical doses than from the higher ones. This implies a reservoir effect involving the upper layers of the stratum corneum. When the trials at 320 $\mu g/cm^2$ were compared, the amount recovered from the skin surface was greater for sulphonamide, the least volatile repellent, than for *m*-deet or ethylhexanediol.

DISCUSSION

In determining percutaneous penetration from the data presented in this paper, two simplifying approximations have to be made. The first is that the distribution and excretion of a compound is the same after both topical and iv administration. The second approximation is that metabolism of a compound occurs secondarily to penetration and is therefore irrelevant. The second approximation is based on the premises that the stratum corneum is the rate-limiting barrier to percutaneous penetration (Scheuplein & Blank, 1971) and that the stratum corneaum is a dead, metabolically inactive membrane. Because of these approximations, the percutaneous penetration values derived here should be regarded as estimates.

The recovery of a compound's radioactive dose in the urine following iv administration provides an estimate of the efficiency of urinary excretion of the compound or its metabolites once it has penetrated the skin and entered the circulation. To calculate the percutaneous penetration, the percentage of applied radioactive dose recovered in the urine following topical application is divided by the fraction of the radioactive dose recovered in the urine following iv administration (Table 6). As expected, the amount of repellent transferred across the skin per unit area increased with increasing chemical dose (Table 6). The mean percentage percutaneous penetration increased slightly at low chemical dose for sulphonamide and *m*-deet, and decreased slightly for ethylhexanediol, but none of these changes was significant at the 95% confidence level (paired comparison using the Student's two-tailed *t* test). However, for sulphonamide (Table 3), the percentage percutaneous penetration for each dog consistently increased with each decrease in chemical dose. The percentage percutaneous penetration may be more sensitive to the chemical dose applied in the case of compounds of low volatility, such as were tested by Wester & Maibach (1976) and Scheuplein & Ross (1974).

The percentage percutaneous penetration of *m*-deet applied at 4 $\mu g/cm^2$ to the hairless dog compares favourably with that reported by Feldmann & Maibach (1970) for the same dose (4 $\mu g/cm^2$) applied to man (16·7 $\pm$ 5·1%). However, additional comparative permeability tests between man and the hairless dog need to be carried out to establish the merit of this animal model for permeability studies.

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ULTRASTRUCTURAL LESIONS OF CLIOQUINOL TOXICITY IN BEAGLE DOGS

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Abstract—Four adult beagle dogs were given clioquinol daily at an oral dose of 200 mg/kg body wt for a period of about 100 days. Three out of the four dogs developed neural disease characterized principally by posterior ataxia. Degenerative neural lesions occurred in each dog and were most prominent in the distal portions of optic-nerve fibres and in the nerve fibres in the dorsal funiculus of the spinal cord. Ultrastructurally, the lesions were characterized by axonal degeneration and axonal loss with secondary alterations in myelin and glial elements. Possible pathogenic mechanisms of clioquinol neurotoxicity are discussed.

INTRODUCTION

Clioquinol (7-iodo-5-chloro-8-hydroxyquinoline), a halogenated hydroxyquinoline compound, was used widely for the treatment of amoebic and nonspecific diarrhoea and as a prophylactic for 'traveller's diarrhoea' until its implication as an aetiological agent in the neurological syndrome of humans, subacute myelo-optico-neuropathy (Kono, 1975). In experimental studies in dogs, clioquinol caused degeneration of the distal aspects of optic-nerve fibres and of the dorsal funiculus of the spinal cord along with mild degenerative changes in other long-fibre tracts of the spinal cord, but did not affect the perpheral nervous system (Heywood, Chesterman & Worden, 1976; Hoover & Carlton, 1980; Krinke, Schaumburg, Spencer, Thomann & Hess, 1979; Worden, Heywood, Prentice, Chesterman, Skerrett & Thomann, 1978). This paper reports the ultrastructural alterations caused by subacute clioquinol intoxication.

EXPERIMENTAL

Four adult (13–15-month old) beagle dogs were given clioquinol (200 mg/kg body wt/day) orally in gelatin capsules for a period of about 100 days. Two beagle dogs served as controls. At the end of the treatment period the dogs were anaesthetized with thiopental and the vascular system was perfused, *via* intracardiac cannulation, with a phosphate-buffered 2% glutaraldehyde-2% paraformaldehyde fixative solution. Autopsy was carried out on each dog following the whole body fixation. One-millimetre cross sections of optic nerves (at the level of the optic canal) and quartered cross sections of the spinal cord (at the levels of the second cervical and the fifth lumbar spinal-cord segments) were collected for ultrastructural examination. The sections were washed in phosphate buffer, transferred to osmium tetroxide for post-fixation osmification, dehydrated in a graded series of acetone solutions and infiltrated and embedded in Epon 812 (Polysciences Inc., Warrington, PA).

Areas of the spinal cord, primarily from the fasciculus gracilis, and the optic nerve were selected for ultrastructural examination from 1-μm sections stained with azure II–methylene blue. Thin sections of selected areas were picked up on 300- or 400-mesh uncoated copper grids, stained using uranyl acetate followed by lead citrate and examined and photographed using a JEOL JEM-100 CX electron microscope.

RESULTS

Three out of the four dogs given clioquinol developed clinical neurological disease observed as early as day 18 of treatment. The affected dogs characteristically had ataxia that primarily involved the hind quarters. However, the fore quarters in two dogs were mildly or moderately affected as well. Leg movements were dysmetric and the hind quarters tended to collapse under body weight. The functions of cranial nerves were normal.

The dogs, both those affected and the one clinically normal, had a sharply defined central wedge-shaped area of degeneration in the dorsal funiculus, primarily involving the rostral aspects of the fasciculus gracilis. The area of degeneration included part or all of the fasciculus cuneatus and also the caudal aspects of the

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dorsal funiculus in more severely affected dogs. Optic-nerve fibres had degenerative changes of varying severity. Retrobulbar areas were minimally or not affected, whereas the more distal aspects of the optic-nerve fibres in the optic tracts frequently had prominent lesions.

Ultrastructurally, the lesions in both the optic nerve and the fasciculus gracilis were characterized by degeneration and loss of axons with associated collapse and degeneration of the myelin sheaths and by astrocytic scarring. The affected axons commonly had contracted and granular electron-dense axoplasm without recognizable organelles (Fig. 1). In some axons, the changes were less severe and consisted of loss of microtubules and neurofilaments with accumulation of electron-dense material (Fig. 2). A few axons contained accumulations of mitochondria, membranous proliferations and electron-dense bodies (Fig. 3) and some axons had accumulations of smooth endoplasmic reticulum, vesicular structures or electron-dense granules (Figs 4 & 5).

Alterations in the myelin sheaths were found only in association with axonal lesions. The myelin sheath and its inner tongue of oligodendrocytic cytoplasm often were morphologically normal in nerve fibres with partial or complete axonal loss (Fig. 6). Some nerve fibres with axonal loss had myelin sheaths that were severely dilated. In a few nerve fibres with axonal loss, the diameter of the nerve fibre was increased and the myelin was thinned. In others, separation of myelin lamellae was accompanied by the accumulation of granular material (Fig. 7).

Oligodendrocytes infrequently had alterations. Rarely an oligodendrocyte, identified by nuclear chromatin clumping and electron-dense cytoplasm, was enclosed by a thin, aberrant myelin sheath. Astrocytic processes were increased in size and number and filled the space between collapsed myelin and degenerated myelin sheaths (Fig. 1). The cytoplasm of astrocytes was increased and in some cells contained a small amount of myelin debris. A few phagocytes were present, usually between astrocytic processes, but occasionally within a myelin sheath. Vascular and connective tissue elements appeared ultrastructurally normal.

DISCUSSION

The changes in the axons of most of the affected nerve fibres were advanced and either the axons were lost or only structureless electron-dense axonal remnants remained. Nerve fibres in various stages of degeneration were found. However, lesions of the myelin sheath were found only in association with axonal alterations. On this basis, it was concluded that clioquinol produced a primary axonal degeneration followed by secondary changes in the myelin. A previous report (Krinke *et al.* 1979) of the ultrastructural neuropathology induced by clioquinol described the axonal changes as a spectrum of nonspecific abnormalities, like those found in our study, but also described were moderate accumulations of 10-nm neurofilaments, a feature rarely observed in our material. Also, no mention was made by Krinke *et al.* of the proliferation of smooth endoplasmic reticulum in altered axons that was occasionally observed in our material. Axonal degeneration with loss of neurofilaments and microtubules and accumulations of floccular electron-dense material and granular debris or the accumulation of vesicles, dense bodies and mitochondria are common reactions of nerve fibres to a variety of insults and occur in Wallerian degeneration (Hirano, 1972).

Other studies have demonstrated that the neurotoxic effects of clioquinol in beagle dogs are found principally in the central nervous system. The peripheral nervous system remained unaffected even when there were moderate to severe degenerative lesions in the central nervous system. (Heywood *et al.* 1976; Hoover & Carlton, 1980; Krinke *et al.* 1979; Worden *et al.* 1978).

An acceptable hypothesis to account for the neurotoxicity of clioquinol must consider the metabolic disturbances affecting the distal axon and the predilection of the central nervous system, especially of the dorsal funiculus and the optic-nerve fibres, for the development of lesions.

Clioquinol accumulates less in the central nervous system than in the retina, spinal ganglia and peripheral nerves (Ogata, Watanabe, Tateishi, Kuroda, Kira, Hasegawa & Otsuki, 1974; Toyokura, Takasu & Mitsuoka, 1975). This suggests that the blood–brain barrier limits the entrance of clioquinol into the central nervous system and that higher concentrations of clioquinol are reached in tissues in the peripheral nervous system where there is a different structural relationship between blood and tissues.

Therefore, the sensitivity of the dorsal funiculus and the optic-nerve fibres for the development of lesions is not explained by a higher affinity of these tracts for the compound. The optic-nerve fibres and the nerve fibres of the dorsal funiculus of the spinal cord both have the cell bodies of their axons outside of the confines of the blood–brain barrier—for the optic nerve in the retina and for the dorsal funiculus in the spinal ganglia—where the vascular relationships to the nervous tissues may be sufficiently different to allow for greater penetration and thus greater concentrations of clioquinol. Thus, the axonopathy caused by clioquiol may be due to a primary effect on the nerve cell body rather than on the axon where the lesion is found.

Many axonopathies appear related to impairment of energy synthesis, which, over extended periods, affects the distal portions of axons but does not produce visible changes in the more proximal portions of axons or in the neuronal cell bodies (Spencer, Sabri, Schaumburg & Moore, 1979). *In vitro* clioquinol uncoupled oxidative phosphorylation in rat liver mitochondria (Hagihara & Yagi, 1975; Yamanaka, Imanari, Tamura & Yagi, 1973). and inhibited transport of proteins in rabbit vagus nerves (McLean & Sjöstrand, 1977a,b). The latter observation was consistent with studies that have demonstrated that fast axoplasmic flow was closely dependent on oxidative phosphorylation (Ochs, 1974). The extent to which the results of these *in vitro* studies apply to the *in vivo* situation remains uncertain and the explanation for restriction of the lesions to the central nervous system is unknown at present.

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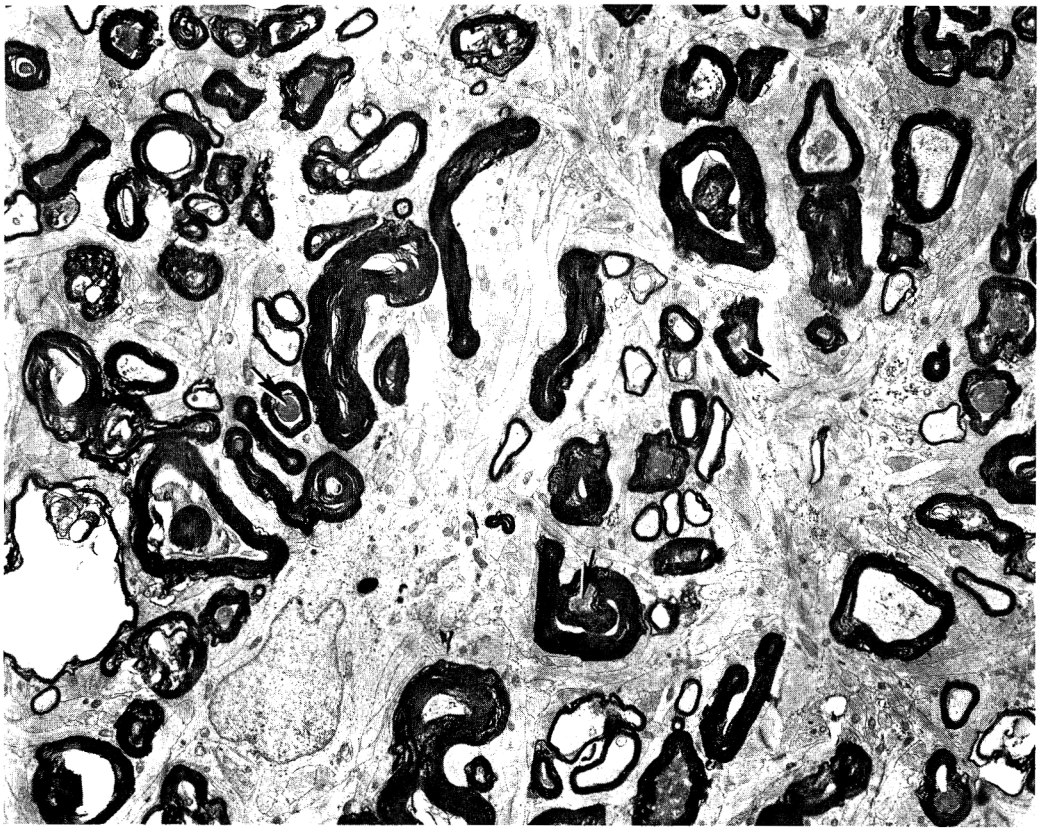

Fig. 1. An optic nerve of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. The nerve has generated axons (arrowed) and collapsed myelin sheaths with increased astrocytic processes. Lead citrate and uranyl acetate × 4000.

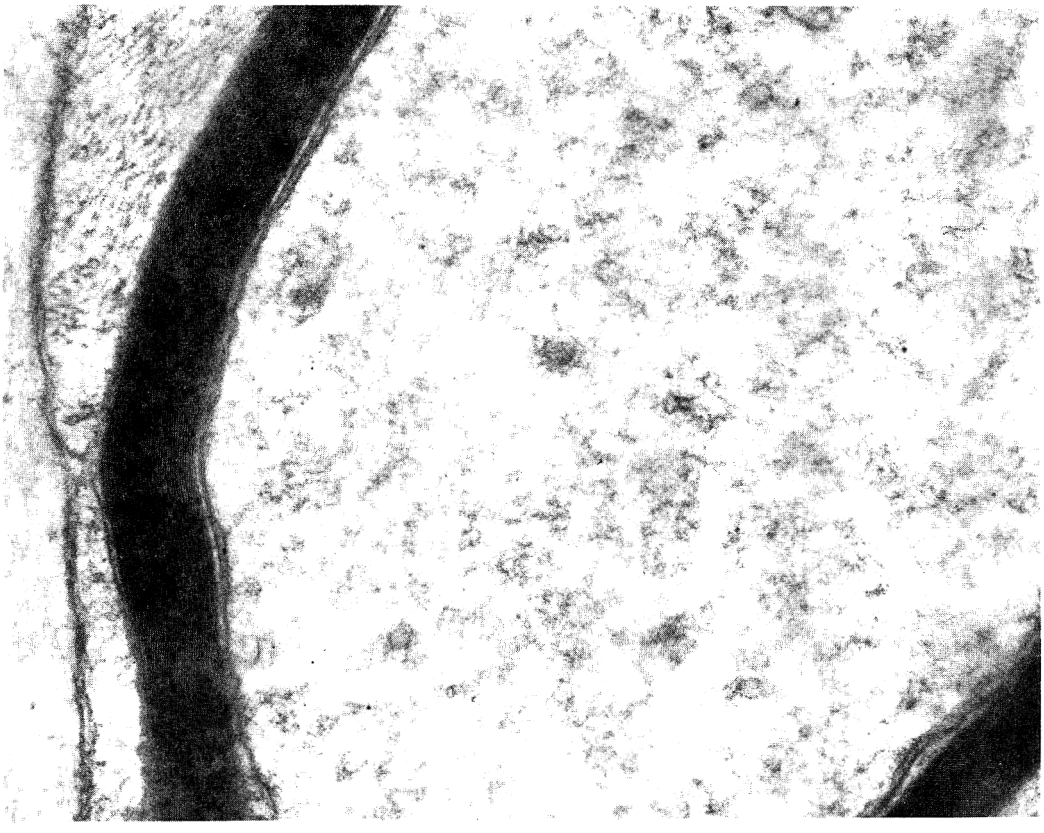

Fig. 2. An axon in the optic nerve of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. The neurofilaments and microtubules have been replaced by flocculent material. Lead citrate and uranyl acetate × 75,000.

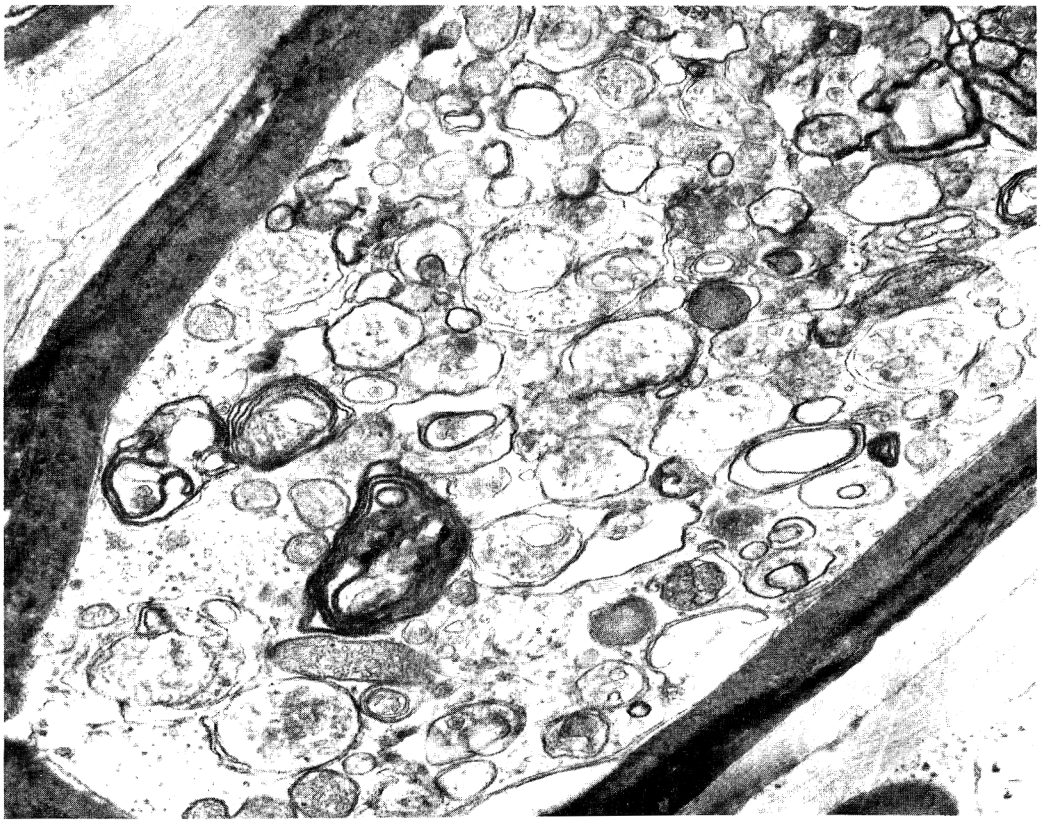

Fig. 3. An axon in the optic nerve of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. The axoplasm is filled with mitochondria, membranous proliferations and electron-dense bodies. Lead citrate and uranyl acetate × 25,000.

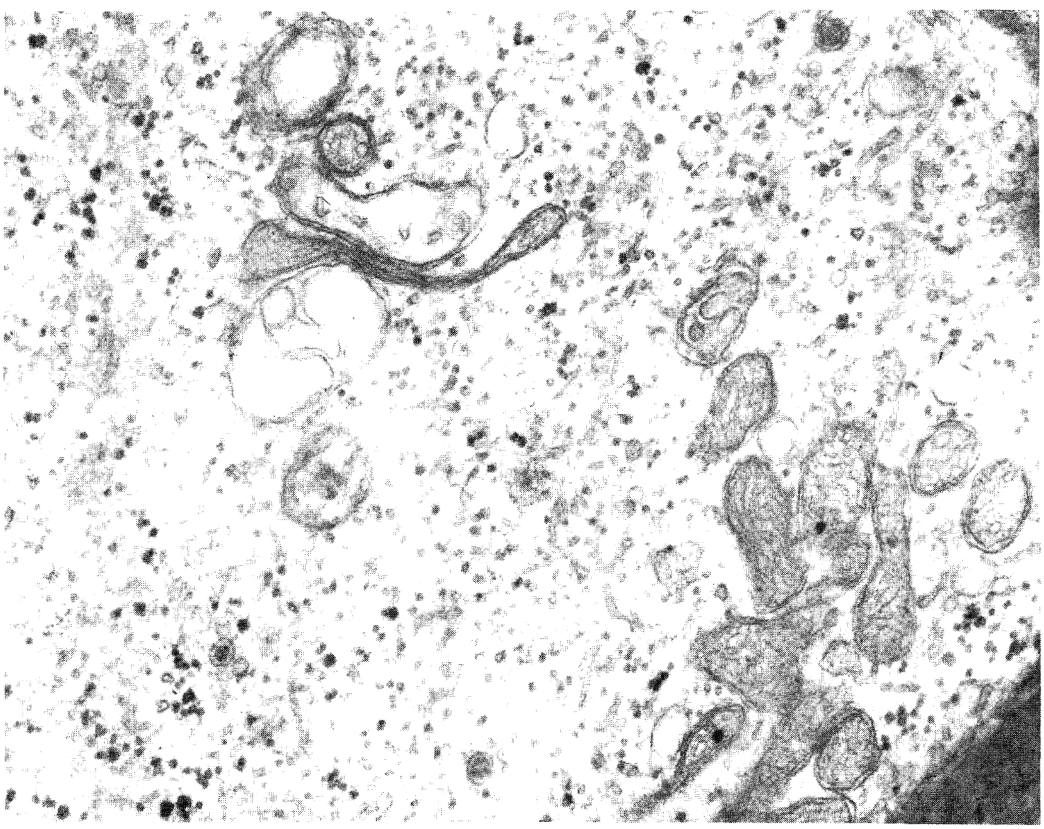

Fig. 4. An axon in the optic nerve of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. Mitochondria, smooth endoplasmic reticulum and electron-dense granules are present in the axoplasm. Lead citrate and uranyl acetate × 52,000.

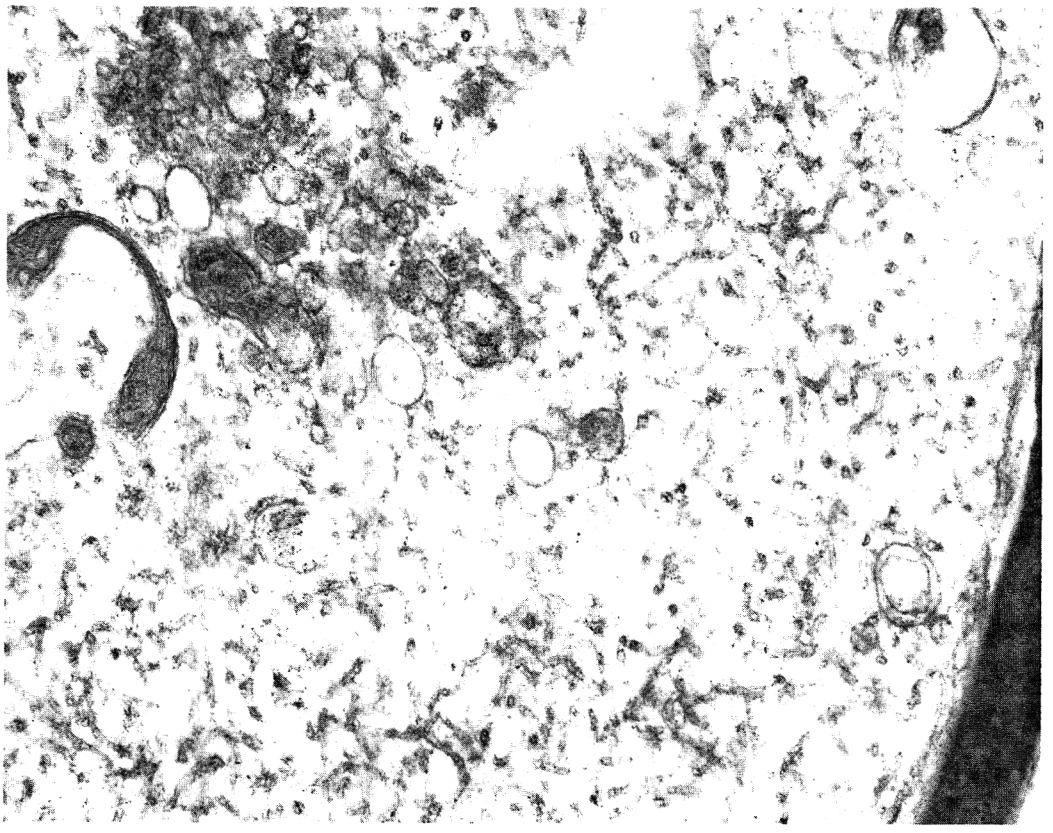

Fig. 5(a).

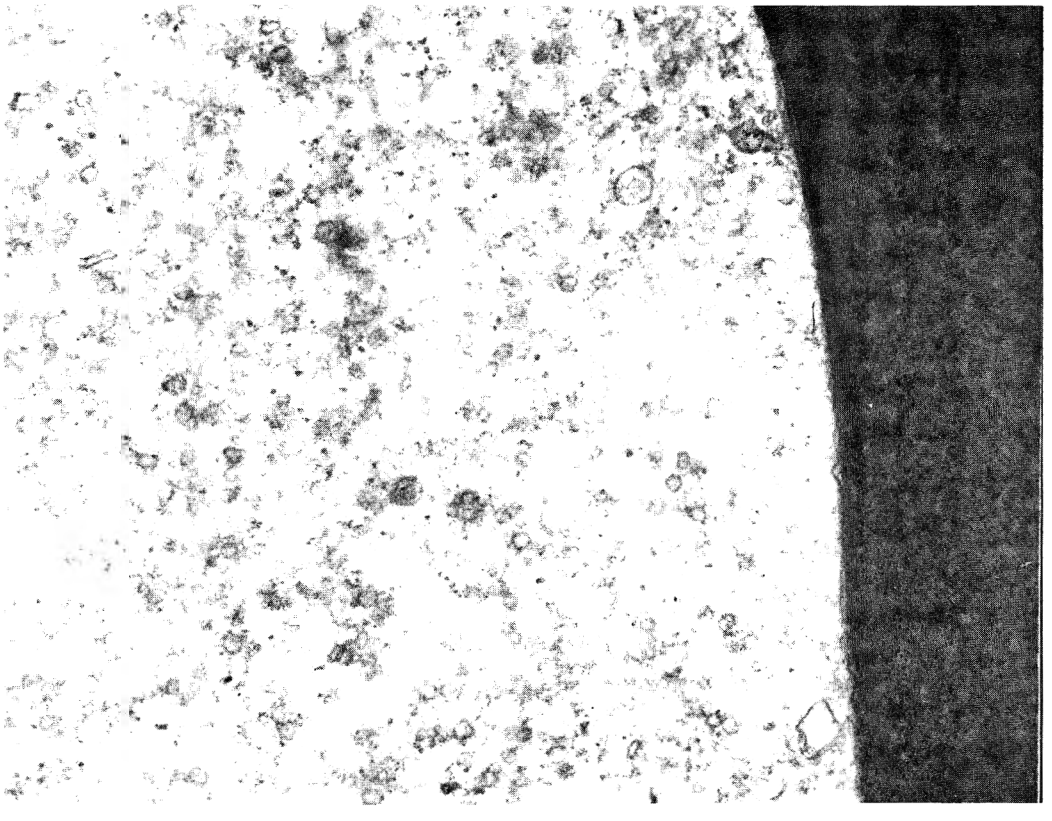

Fig. 5(b).

Fig. 5. An axon in the dorsal funiculus (fifth lumbar spinal-cord segment) of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. (a) Microtubules and neurofilaments are absent and axoplasmic proliferations of smooth endoplasmic reticulum are present. (b) The axoplasm contains small vesicular structures, neurofilaments and flocculent electron-dense material. Lead citrate and uranyl acetate × 52,000.

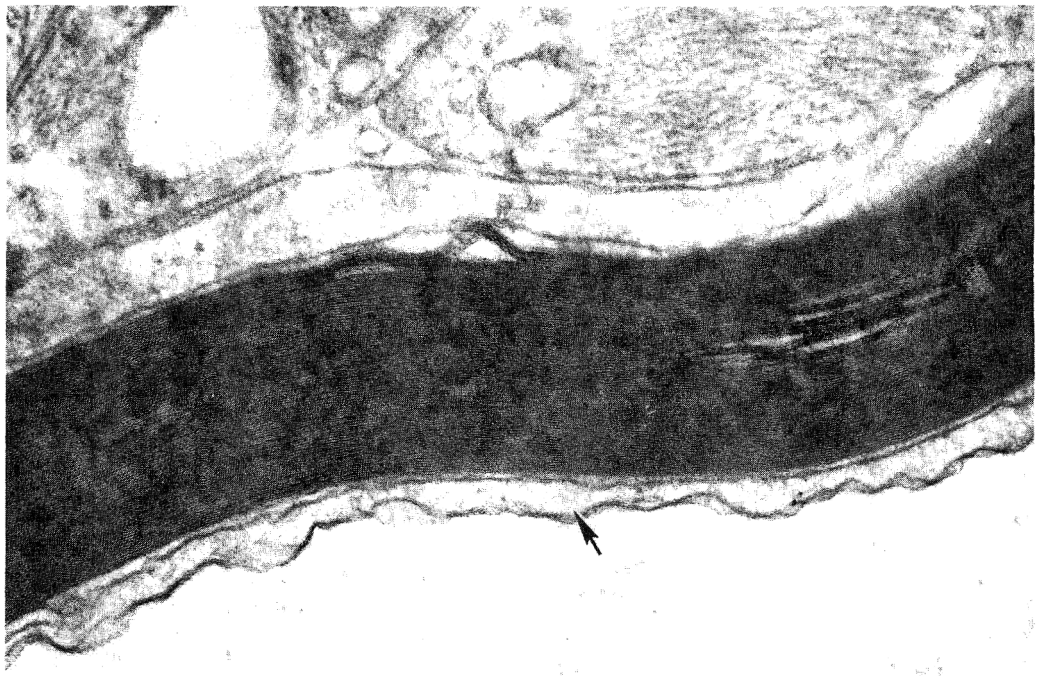

Fig. 6. Nerve fibre in the dorsal funiculus (fifth lumbar spinal-cord segment) of a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. The axon is absent and the inner tongue of the myelin sheath (arrowed) is intact. Lead citrate and uranyl acetate ×81,000.

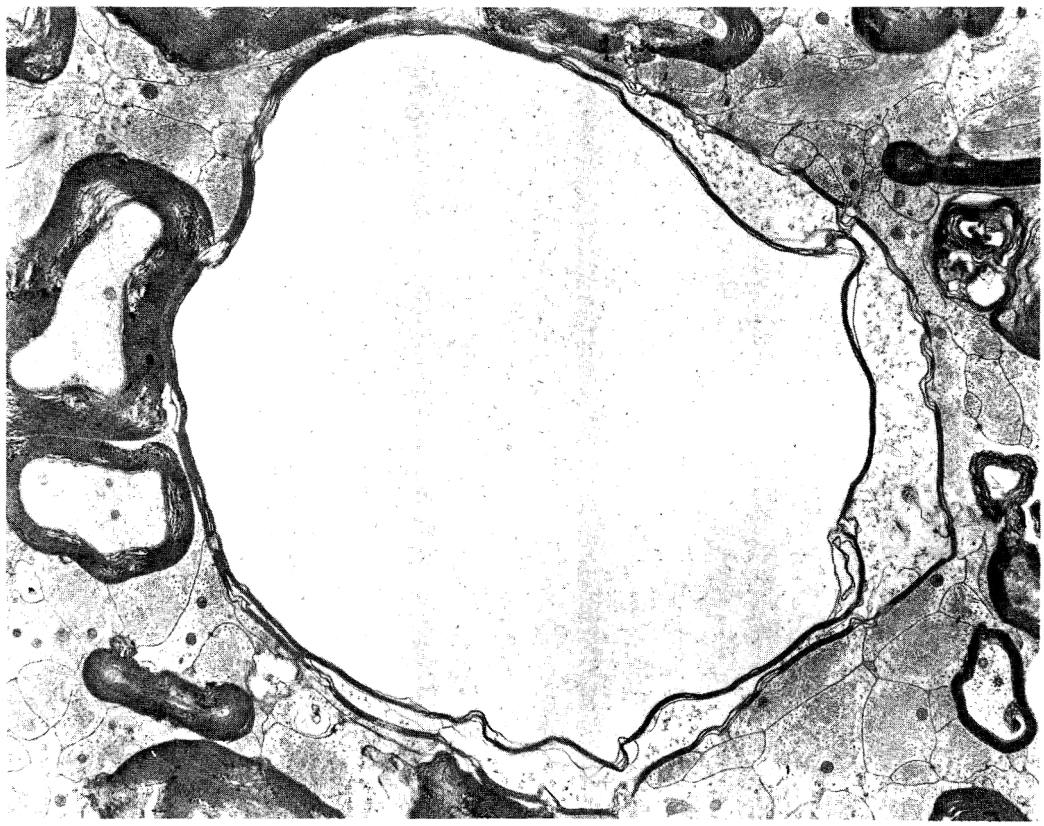

Fig. 7. Fasiculus gracilis (second cervical spinal-cord segment) from a beagle dog given 200 mg clioquinol/kg body wt/day for about 100 days. The central nerve fibre is increased in diameter, has no axon and the myelin sheath is attenuated and the lamellae in the myelin sheath are separated. Lead citrate and uranyl acetate ×8000.

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A SULPHITE-OXIDASE-DEFICIENT RAT MODEL: METABOLIC CHARACTERIZATION

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Abstract—Rats were made deficient in sulphite oxidase by the administration of a high-tungsten/low-molybdenum regimen as described first by Johnson *et al.* (*J. biol. Chem.* 1974, **249**, 859). The specific protocol used resulted initially in an exponential decrease in hepatic sulphite-oxidase activity with a half-life of 4 days and an eventual steady-state enzyme level approximately 1% of the normal adult level. A clear inverse relationship was demonstrated between hepatic sulphite-oxidase activity and tissue and/or urine concentrations of sulphite and of two sulphite metabolites, *S*-sulphonate ($RS-SO_3^-$) and inorganic thiosulphate ($S_2O_3^{--}$). As rat tissues became depleted of sulphite-oxidase activity, sulphite produced endogenously from the catabolism of sulphur-containing amino acids gradually became apparent. The first chemical evidence of an increased systemic concentration of sulphite occurred when the sulphite-oxidase activity had declined to about 7% of the normal adult level; at this point, slight but significant increases in urinary $S_2O_3^{--}$ and $RS-SO_3^-$ concentrations were observed. The additional decline of sulphite oxidase to 1% of the normal level resulted in substantial increases in the excretion of both of these metabolites. In addition, large increases in aortic $RS-SO_3^-$ concentrations relative to pretreatment levels and smaller increases in plasma and pinna $RS-SO_3^-$ concentrations were observed as the rats approached a steady-state enzyme level. The ability of these sulphite-oxidase-deficient rats (1% of normal activity) to clear sulphite following intragastric sulphite administration was compared with that of normal rats. The results showed that deficient rats were much less efficient at this process and thus required lower exogenous doses to produce equivalent systemic exposures.

INTRODUCTION

Sulphite/bisulphite (hereafter referred to as sulphite, SO_3^{--}) is generated endogenously in mammalian tissues from the catabolism of sulphur-containing amino acids. Tissue SO_3^{--} can also result from exposure to exogenous sources such as atmospheric sulphur dioxide and foods and beverages containing SO_3^{--} salts as additives. Under usual circumstances, the endogenously generated load is estimated to be several orders of magnitude greater than that from exogenous sources (Institute of Food Technologists and Committee on Public Information, 1976).

Sulphite is a very reactive ionic species which interacts with molecules of biological importance in potentially toxic reactions. For example, SO_3^{--} has been shown to react *in vitro* with cytosine and uracil in DNA and RNA, respectively, and to damage DNA chains presumably by a free-radical mechanism (Shapiro, 1977). Sulphite is also capable of thiamine cleavage (Williams, Waterman, Keresztesy & Buchman, 1935) and *in vivo* lysis of certain protein disulphide bonds (Gunnison & Farruggella, 1979). In mammals, tissues are protected from exposure to SO_3^{--} primarily by its direct oxidation to the relatively non-toxic sulphate ion. This metabolic step is catalysed by sulphite oxidase, a mitochondrial enzyme containing functional molybdenum (Mo) and present at high levels in the liver and also in lesser concentrations in most tissues of the body (Cohen, Betcher-Lange Kessler & Rajagopalan, 1972; Cohen, Fridovich & Rajagopalan, 1971; MacLeod, Farkas, Fridovich & Handler, 1961).

There are significant differences among species in their sulphite-oxidase activity. Most notable is the difference between the rat and man, with the latter reported to possess only about 5–10% of the hepatic activity of the rat (Johnson & Rajagopalan, 1976a,b). Therefore, although the rat has been used predominantly in the past for evaluation of SO_3^{--} toxicity, it is our contention that this species may not be the most appropriate model available for the prediction of toxicity in man.

Johnson, Cohen & Rajagopalan (1974) have demonstrated that rat tissues can be depleted of sulphite-oxidase activity by maintaining animals on a regimen high in tungsten (W) and low in Mo. They suggested that rats thus treated might be used as a model for human sulphite-oxidase-deficiency disease, although early attempts to duplicate the physiological signs of this disease were not conclusive (Johnson & Rajagopalan, 1976b). The human disease stems from an apparently rare genetic disorder characterized by congenital sulphite-oxidase deficiency and manifested by severe mental and physical retardation. The main object of the work reported here is to define, and characterize metabolically, a sulphite-oxidase-deficient rat model, utilizing the concept introduced by Rajagopalan and his coworkers at Duke University Medical Center (Johnson *et al.* 1974). Our ultimate objective is to use this model for the evaluation of potential human SO_3^{--} toxicity.

In this paper, we demonstrate the quantitative alteration of SO_3^{--} metabolism as a function of sulphite-oxidase activity. Data from previous studies suggest specific SO_3^{--} metabolites that should appear

as sulphite oxidase is depleted. For example, in cases of human sulphite-oxidase-deficiency disease, inorganic thiosulphate ($S_2O_3^{--}$) and cysteine *S*-sulphonate (Cys–S–SO_3^-) as well as SO_3^{--} itself are excreted into the urine in large amounts, and the two latter substances can also be detected in the plasma. In normal individuals, both SO_3^{--} and Cys–S–SO_3^- are undetectable. In addition, experiments in this laboratory on normal mammals have demonstrated that, in several tissues, elevated levels of *S*-sulphonate (RS–SO_3^-) metabolites can result from the administration of exogenous SO_3^{--} and sulphur dioxide (Gunnison & Farruggella, 1979; Gunnison & Palmes, 1978; Gunnison, Zaccardi, Dulak & Chiang, 1981). These studies, then, suggest specific compounds, tissues and fluids that can be monitored to detect changes in SO_3^{--} metabolism.

EXPERIMENTAL

Experimental model. Virgin female Wistar rats, weighing approximately 200–225 g and purchased from Marland Breeding Farms, Hewitt, NJ, were fed *ad lib.* either Purina Lab Chow (PLC; Ralston Purina Co., St. Louis, MO) or a normal-protein diet (NP) low in Mo (ICN Nutritional Biochemicals, Cleveland, OH). They were housed in hanging wire-mesh cages at 20–23°C with a 12-hr light/dark cycle. The NP diet was assayed colorimetrically for Mo content (Association of Official Analytical Chemists, 1975) by Raltech Scientific Services, Inc., Madison, WI, and was found to contain 0·058 ppm Mo. The Mo concentration in a sample of PLC was determined to be 2·0 ppm. Rats given the low-Mo NP diet were made sulphite-oxidase deficient by concurrent addition of sodium tungstate to their drinking-water, as described previously by Johnson *et al.* (1974). The specific protocol consisted of addition of 200 ppm W as sodium tungstate to glass-distilled water for 28 days, followed by addition of W at a level of 100–120 ppm for the duration of the experiment (up to 63 days). This protocol is referred to throughout as the 'high-W/low-Mo' regimen and the experimental group is designated as either NP/W or sulphite-oxidase-deficient. Four separate shipments of rats, referred to as 'series', were received sequentially and subjected to this regimen. Rats in two of these series were mated after approximately 42 days of treatment (t_{42}) and used in a teratological investigation, the results of which will be reported in a later publication. Tissues from some of these pregnant rats were used in the metabolism experiments reported here.

Three other experimental groups used in this study each received a regimen designed to maintain normal tissue levels of sulphite oxidase. Two of these groups (NP and PLC) were given tap-water and the NP or PLC diet, respectively. The third group (NP/W/Mo) received a regimen identical to the NP/W group except that 4 ppm Mo, as sodium molybdate, was included in the drinking-water. These three control groups were run concurrently with the NP/W group.

Tissue and urine collection. Rats were killed by CO_2 asphyxiation and their livers, aortas and ear tips (distal third of the pinna) were removed and stored at −90°C until analysed. Whole blood was collected by heart puncture just prior to the killing, using either heparin or sodium citrate as an anticoagulant, and the plasma was stored at −90°C. The liver was used for assay of sulphite-oxidase activity and the other tissues were analysed for *S*-sulphonate (RS–SO_3^-) compounds.

Urine devoid of faecal pellets and only slightly contaminated by food was collected from rats placed in metabolism cages for approximately 16 hr, including the entire 12-hr dark portion of the light/dark cycle. Urine was collected at the end of this period, centrifuged for approximately 5 min in a clinical centrifuge to separate any contaminating feed, and stored at −90°C. The mean time during which urine remained at room temperature was considered to be 8 hr. The urine from three to six rats, all subjected to the same regimen for the same number of days (i.e. same 't' time) was combined for later analysis.

S-Sulphonate analytical method, general considerations. The fundamental steps of the analytical method for the determination of RS–SO_3^- are the same regardless of the tissue or fluid involved. Modifications are made only to accommodate differences in the physical or chemical characteristics of the material being analysed. Following tissue preparation, the first step consists of incubation of the sample for 1–1·5 hr at 36 ± 1°C under nitrogen in 0·07–0·1 M-potassium cyanide at a pH above 10. Under these conditions, cyanide attacks the disulphide bond of RS–SO_3^- releasing a stoichiometric quantity of SO_3^{--}, which is subsequently determined quantitatively by a modification of the method of West & Gaeke (1956) and used to calculate the amount of RS–SO_3^- originally in the sample. Obviously free SO_3^{--} present initially in the sample would give a positive interference. Prior to analysis of the cyanolysed SO_3^{--}, it is usually necessary to separate it from the bulk of the soluble and insoluble protein in the incubation mixture, either by dialysis or by precipitation of protein. In the subsequent analysis of dialysate or supernatant for SO_3^{--}, the use of sodium tetrachloromercurate is crucial since it not only stabilizes the SO_3^{--} but, more importantly, reduces the positive interference of excess cyanide, apparently by formation of a stable complex.

Plasma, aorta and pinna RS–SO_3^- analyses. Detailed methods have previously been published for the analysis of RS–SO_3^- in plasma (Gunnison & Palmes, 1973) and in the aorta (Gunnison & Farruggella, 1979). The method for the determination of RS–SO_3^- in the pinna is essentially identical to that for the aorta except that the pinna tissue need not be cleaned and lyophilized to obtain a dry weight since, in this case, accurate fresh weights can be obtained.

Urinary RS–SO_3^- analysis. A 10-ml aliquot of pooled urine was stirred for 30 min at room temperature with 20 ml XAD-2 resin (Mallinckrodt Inc., St. Louis, MO), prewashed as described by Yamasaki & Ames (1977). The mixture was suction filtered and washed with 20 ml water. The combined filtrate and washings were then lyophilized and reconstituted with water to 10 ml, and 1-ml aliquots were analysed for RS–SO_3^- content. The usual RS–SO_3^- analytical method was modified by addition of 50 μmol EDTA and sufficient base (approximately 0·2–0·25 mequiv. NaOH) to raise the pH above 10 for the cyanolysis step. Following incubation, 0·45–0·55 mequiv. HCl

and 0·18 mmol sodium tetrachloromercurate were added and the sample volume was brought to 5 ml. A slight precipitate that usually formed was removed by centrifugation and an aliquot of the supernatant was analysed for SO_3^{--} against a matched blank prepared by addition of 0·02 ml 0·1% H_2O_2 to an identical supernatant aliquot. The exact amounts of base and acid required for pH adjustment during the analysis varied slightly among the pooled urine samples and were determined by titration prior to analysis. (The two critical pH values in the analysis were a pH greater than 10·0 during cyanolysis and a pH between 1·4 and 1·6 for proper colour development in the final step.)

Urinary $S_2O_3^{--}$. Samples of pooled urine up to 10 ml in volume and containing less than 3 μmol $S_2O_3^{--}$ were made 20 mM in exogenous monosodium hydrogen phosphate and analysed for $S_2O_3^{--}$ content according to the method of Sörbo & Öhman (1978), with two major modifications. Amberlyst A 21 ion-exchange resin (Gallard–Schlesinger Chemical, Carle Place, NY) was ground lightly with a mortar and pestle and used in place of Lewatit MP 7080 resin (B. Sörbo, personal communication, 1979). The second modification involved a change in the pH for the conversion of $S_2O_3^{--}$ to thiocyanate (SCN^-) from 10·5 to 8·25. The pH 10·5 eluate obtained from the first Amberlyst column was titrated with sulphuric acid to pH 8·25 before addition of the KCN and $CuCl_2$ reagents for effecting the conversion to SCN^-. This mixture was then passed through the second Amberlyst column (approximately 0·7 × 3·5 cm) which had previously been equilibrated to pH 8·25 with the pH-adjusted ammonia/ammonium sulphate buffer. The column was washed with 30 ml ammonia/ammonium sulphate buffer (pH 10·5) which had been diluted with water (2 parts buffer:1 part water) and the analysis was completed without additional modification. A slight green precipitate sometimes formed during the conversion to SCN^- at pH 8·25; this was redissolved in the pH 10·5 buffer and applied to the column.

Urinary creatinine analysis. A colorimetric procedure utilizing the Jaffé reaction was used for the analysis of creatinine in pooled urine samples (Oser, 1965).

Plasma and urinary SO_3^{--} *analyses.* Plasma SO_3^{--} concentrations were determined as described by Gunnison & Palmes (1976). The same method was modified for urinary SO_3^{--} by inclusion of 0·09 mequiv. HCl in the analysis of a 0·1-ml sample.

Assay of hepatic sulphite oxidase. Hepatic sulphite-oxidase activity was determined spectrophotometrically essentially as described by Johnson *et al.* (1974) and by Kessler & Rajagopalan (1972), except that liver homogenization was performed with a Polytron (Brinkman Instruments, Westbury, NY) and sodium deoxycholate was added to the homogenate to a final concentration of approximately 0·1%. Sulphite-oxidase activity is expressed in this paper as specific activity (units of activity/mg of protein) with a unit of activity defined as a change in optical density of 0·1/min in a 1-cm light path.

Protein determination. The protein concentrations of plasma and of liver homogenates were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.

Carotid cannulation and administration of exogenous SO_3^{--}. The right carotid arteries of female sulphite-oxidase-deficient and normal rats weighing approximately 230–280 g were cannulated under ether anaesthesia and the cannulae were externalized dorsally. Beginning at 16.00 hr on the day of cannulation, rats were fasted for 18 hr prior to the administration of 2 ml of an aqueous solution of sodium metabisulphite by gastric intubation. Following dosing, plasma SO_3^{--} concentrations were determined in 0·2-ml heparinized blood samples collected sequentially from the cannulae.

RESULTS

Sulphite-oxidase activity

Figure 1 illustrates the effect of manipulation of W and Mo intakes on the hepatic sulphite-oxidase activity of adult female rats. Enzyme activity was exponentially reduced in rats maintained on the high-W/low-Mo regimen during the first 28 days of treatment when the ratio by weight of W to Mo intake was approximately 5800 to 1, exclusive of any W present in the diet. Under these conditions, W atoms prevent the incorporation of Mo atoms into newly synthesized sulphite-oxidase molecules, resulting in the production of non-functional enzyme molecules and a net disappearance of sulphite-oxidase activity (Johnson *et al.* 1974). The rate of disappearance was first order over the 28-day period, and was characterized by a 4-day half-life. This is a similar disappearance rate to that observed by Cohen, Drew, Johnson & Rajagopalan (1973) in rats maintained on the same low-Mo diet with the addition of only 100 ppm W to the drinking-water. Further, in our laboratory, a group of rats given 400 ppm W in their drinking-water demonstrated essentially the same disappearance kinetics over the first 28 days. Thus, under this range of conditions, the limiting factor for disappearance of activity appears to be the rate of turnover of sulphite-oxidase protein. Eventually a steady-state level of sulphite-oxidase activity is attained which depends on the ratio of W to Mo intake. After t_{28}, when the concentration of W in their drinking-water was decreased from 200 ppm to 100–120 ppm (W:Mo intake, 3100:1) the rats in the NP/W group maintained a reasonably steady-state enzyme level of approximately 0·04 specific activity or about 1% of the mean adult female level. Although this experiment lasted only 63 days, it is assumed that the steady-state enzyme level could have been maintained for a considerably longer period of time. In a similar experiment in which rats were transferred on day 28 to a drinking-solution containing 50 ppm W (W:Mo intake, 1450:1), a steady-state enzyme level approximately 60% higher was observed. In a third group of rats given 400 ppm W in the drinking-water (W:Mo intake, 11,600:1), the sulphite-oxidase level decreased below the limit of sensitivity of the analytical method (specific activity less than 0·01).

Rats in the NP group consuming the low-Mo diet without W addition maintained levels of sulphite oxidase comparable to those in the PLC group (Fig. 1). Thus, addition of W was essential for the disappearance of sulphite-oxidase activity. However, the effect

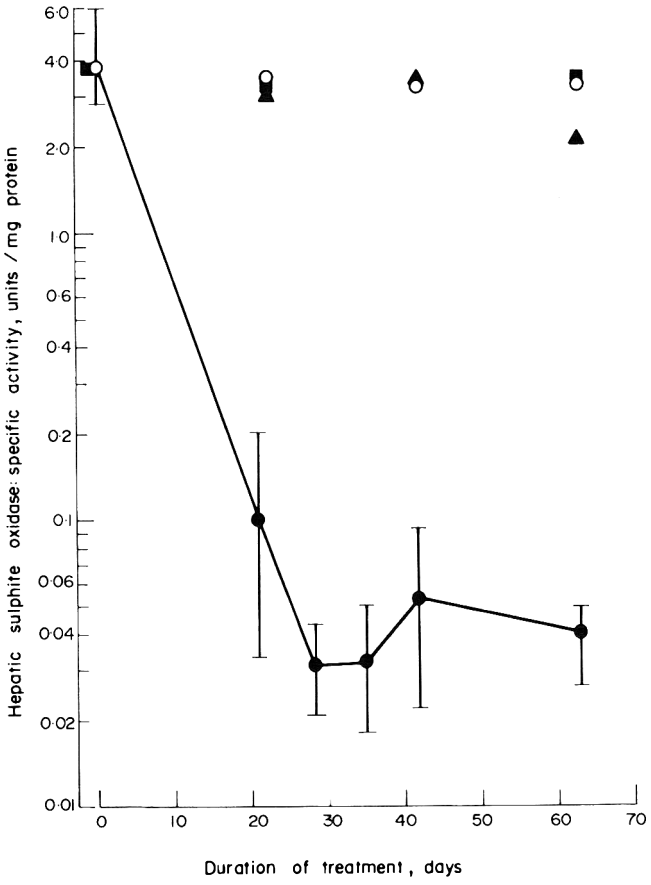

Fig. 1. Hepatic sulphite-oxidase activity as a function of time in female rats maintained on the NP/W (●), NP (○), PLC (■) or NP/W/Mo (▲) regimens identified under 'Experimental' (first paragraph). Values are means of measurements from 3–9 rats and dispersion, where shown, represents the observed range.

of W in the NP/W group was almost completely eliminated by the concurrent addition of 4 ppm Mo (NP/W/Mo group), which reduced the W:Mo intake ratio to 50:1 during the first 28 days of treatment and to 25:1 thereafter (Fig. 1).

General health of animals

The general health and toxic signs of the sulphite-oxidase-deficient and accompanying control rats will be dealt with in detail in a subsequent publication. For the purposes of this biochemical description of the model, it is sufficient to record that there were no consistent overt differences in the general health of sulphite-oxidase-deficient and normal rats.

Evaluation of the analytical method for determining urinary RS–SO_3^- metabolites

Cysteine *S*-sulphonate (Cys–S–SO_3^-) was added to urine collected from rats in the NP group (t_0 or control urine) to final concentrations of approximately 60, 120 and 240 nmol/ml urine (approximately equivalent to nmol/mg creatinine), and the spiked urine was analysed directly for RS–SO_3^- compounds without prior treatment with XAD resin. Recovery of the added Cys–S–SO_3^- was 0, 4 and 77% respectively. When spiked urine was treated with XAD resin prior to RS–SO_3^- analysis (usual method), recovery was greatly improved (Table 1). The analysis of each spiked urine pool was replicated 4–6 times following XAD-resin treatment and all replicates were weighted equally in the calculation of the means (±SD). The mean recovery of Cys–S–SO_3^- added to t_0 urine was 64% at the concentration of approximately 20 nmol/ml and greater than 90% when the spike concentration was increased to 60 nmol/ml. Omission of EDTA in these analyses resulted in a decrease in recovery of approximately 10%; however, EDTA also caused a 10–20% negative interference in the analysis of the Cys–S–SO_3^- standard. Thus, the optical density readings observed in the analysis of urine spiked with Cys–S–SO_3^- were approximately the same whether or not EDTA was added. Cysteine *S*-sulphonate recovery from t_0 urine was not significantly affected by storage at room temperature (20–23°C) for 24 hr or at −90°C for at least 137 days (Table 1). Also, recovery of Cys–S–SO_3^- added at the 20 nmol/ml level to urine collected from rats in the NP/W group 5 and 14 days after initiation of the regimen (t_5 and t_{14} samples) was 74 and 97%, respectively. Thus, it appears that interference in the determination of RS–SO_3^- in urine decreased with both increasing concentration of RS–SO_3^- and increasing time of treatment of rats with

Table 1. *Evaluation of urinary S-sulphonate analysis: recovery of cysteine-S-sulphonate added to urine*

Urine collection			Cys–S–SO_3^- added (nmol/ml)	Storage conditions		Recovery of Cys–S–SO_3^-‡ (%)
Group*	t-Time†	No. of pools		Time (days)	Temp. (°C)	
NP	0	2	20	0§	—	64 ± 21
		2	60	0§	—	94 ± 10
		2	60	1	22	87 ± 13
		1	60	33	−90	106 ± 11
		1	60	123	−90	102 ± 4
NP/W	5	2	20	0§	—	74 ± 18
	14	1	20	0§	—	97 ± 12
	35	1	60	0§	—	103 ± 9

Cys–S–SO_3^- = Cysteine *S*-sulphonate

*The regimens on which the NP (control) and NP/W (sulphite-oxidase-deficient) groups were maintained are identified under 'Experimental' (first paragraph).

†Days from start of treatment.

‡Analysis of each spiked sample of pooled urine was replicated 4–6 times following XAD-resin treatment; results are expressed as means ± 1 SD.

§Samples were spiked with Cys–S–SO_3^-, treated with XAD resin and analysed.

the high-W/low-Mo regimen. Only urine samples collected from rats in the NP group, or at t-times below 14 days in the NP/W group, were corrected for analytical efficiency.

Evaluation of the analytical method for determining urinary $S_2O_3^{--}$

The efficiency of the $S_2O_3^{--}$ analytical procedure of Sörbo & Öhman (1978) in our hands was only 20–30%, although the authors reported 65% (comparisons made against a SCN^- standard curve). We determined that a major fraction of the analytical loss resulted from failure of $S_2O_3^{--}$ to convert to SCN^- in the pH 10·5 buffer and, therefore, modified the procedure as described, improving efficiency to approximately 50–60%. Analytical efficiency was not affected by the presence of SO_3^{--}.

The recovery of $S_2O_3^{--}$ added to urine collected from rats in the NP group (t_0 urine) and from rats in the NP/W group was determined under a range of storage conditions (Table 2). In each case, the amount of $S_2O_3^{--}$ added was 1·0 μmol per urine sample. Recovery from control urine samples averaged 61% when analysis was carried out immediately after

Table 2. *Evaluation of urinary thiosulphate analysis: recovery of thiosulphate added to urine*

Urine collection			Storage conditions		Recovery of $S_2O_3^{--}$‡ (%)
Group*	t-Time†	No. of pools	Time	Temp. (°C)	
NP	0	4	0	—	61 ± 13
		2	15 days	−90	52 ± 2
		1	56 days	−90	45
		1	85 days	−90	46
		4	24 hr	22	21 ± 3
NP/W	6–9	1	8 hr	22	30
	14	1	8 hr	22	44
	21	1	8 hr	22	56
	35	1	8 hr	22	58
	42	1	8 hr	22	50

$S_2O_3^{--}$ = Inorganic thiosulphate

*The regimens on which the NP (control) and NP/W (sulphite-oxidase-deficient) groups were maintained are identified under 'Experimental' (first paragraph).

†Days from start of treatment.

‡1·0 μmol $S_2O_3^{--}$ was added to each sample. Where possible, results are means ± 1 SD.

addition of $S_2O_3^{--}$, and 21% after storage of the spiked urine at room temperature (20–23°C) for 24 hr. Collection of urine in thymol–isopropanol preservative (Naftalin & Mitchell, 1958) did not prevent this disappearance at room temperature. However, storage at −90°C appeared to stabilize the $S_2O_3^{--}$ in urine for up to 85 days.

Since the mean time during which urine remained at room temperature during collection was 8 hr, it was necessary to determine both the loss of $S_2O_3^{--}$ in urine under these conditions and the efficiency of the analytical method, in order to calculate the amount of $S_2O_3^{--}$ excreted in a urine sample. These two correction factors were estimated together by determination of the $S_2O_3^{--}$ recovery from an aliquot of urine containing added $S_2O_3^{--}$, after storage at room temperature for 8 hr. Recovery of this $S_2O_3^{--}$ spike, which was influenced by losses both during storage and in the analytical process, was calculated by subtraction of the endogenous $S_2O_3^{--}$ content determined in a second aliquot from the same urine pool held under identical storage conditions. This factor was then used to correct the $S_2O_3^{--}$ content determined in a third aliquot of the urine not stored at room temperature, thus obtaining a value for $S_2O_3^{--}$ excreted. It is apparent from Table 2 that recovery of exogenous $S_2O_3^{--}$ from urine collected from the NP/W group increased as the time of treatment with the high-W/low-Mo regimen increased up to t_{21}, after which recovery remained reasonably consistent at approximately 50–60%.

Tissue and urinary SO_3^{--} metabolites

As sulphite-oxidase activity was depleted in the NP/W group, concentrations of the SO_3^{--} metabolites RS–SO_3^- and $S_2O_3^{--}$ were monitored in several tissues and in urine to provide quantitative data on the effects of sulphite-oxidase activity on the metabolism of SO_3^{--} generated endogenously from dietary sulphur-containing amino acids. The calculated intake of cysteine and methionine in the NP/W group was 0·14 and 0·34 mmol/kg/day respectively. Similar monitoring was also done with control groups (normal sulphite-oxidase activity), in which sulphur-containing amino acid intake was essentially identical to that of the NP/W group. The results of these experiments are presented in Figs 2 and 3. In conjunction with Fig. 1, they provide a concise picture of the relationship between sulphite-oxidase activity and the accumulation and excretion of certain SO_3^{--} metabolites.

Comparison of Fig. 2a with Fig. 1 clearly illustrates the overall inverse relationship between aortic RS–SO_3^- concentration and sulphite-oxidase activity. Although initially little increase in aortic RS–SO_3^- is seen in the NP/W group as sulphite-oxidase activity decreases, there is a rapid rise in RS–SO_3^- concentration at approximately t_{21} followed by stabilization beginning at t_{28}, indicating attainment of equilibrium between the rate of endogenous SO_3^{--} generation and RS–SO_3^- concentration. This state of equilibrium coincides with the steady-state level of enzyme activity observed between t_{28} and t_{63} (approximately 1% of the normal adult female activity). The aortic RS–SO_3^- concentration during this period is 5–6 times higher than the normal endogenous concentration of the NP group (dotted line in Fig. 2a). The endogenous RS–SO_3^- concentrations in the NP, PLC and NP/W/Mo groups are all similar and exhibit no significant fluctuation during the course of the experimental period, an observation consistent with the levels of sulphite-oxidase activity in these animals (Fig. 1).

The pattern of RS–SO_3^- concentration in the plasma (Fig. 2b) is similar to that observed in the aorta, although there is more variability among data points of the NP/W group during the period of steady-state enzyme levels (t_{28}–t_{63}). Also, the relative increase in RS–SO_3^- is only about 2–3 times the mean endogenous level observed in the NP group (dotted line, Fig. 2b). A low concentration of plasma SO_3^{--} was frequently detected during the steady-state period when sulphite-oxidase activity was reduced to 1% of the normal value. For example, in 18 rats (all tested at

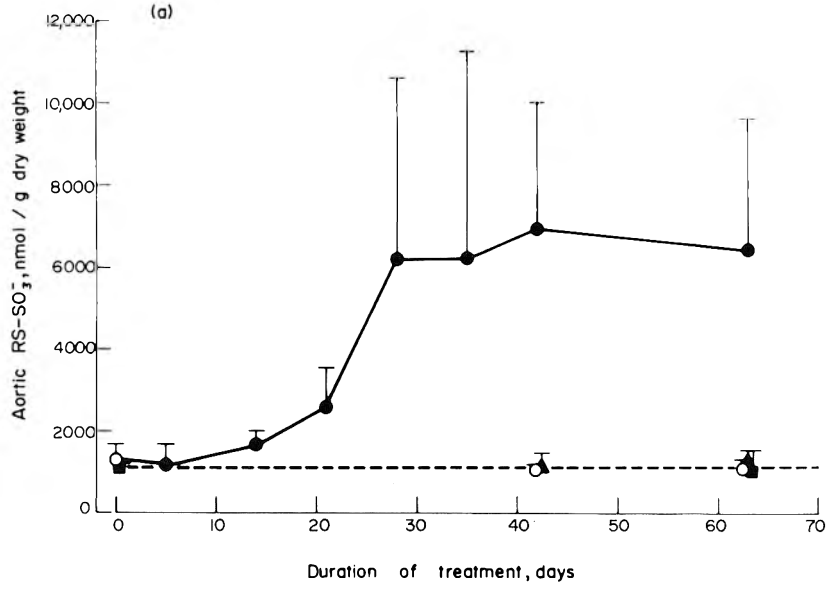

Fig. 2(a).

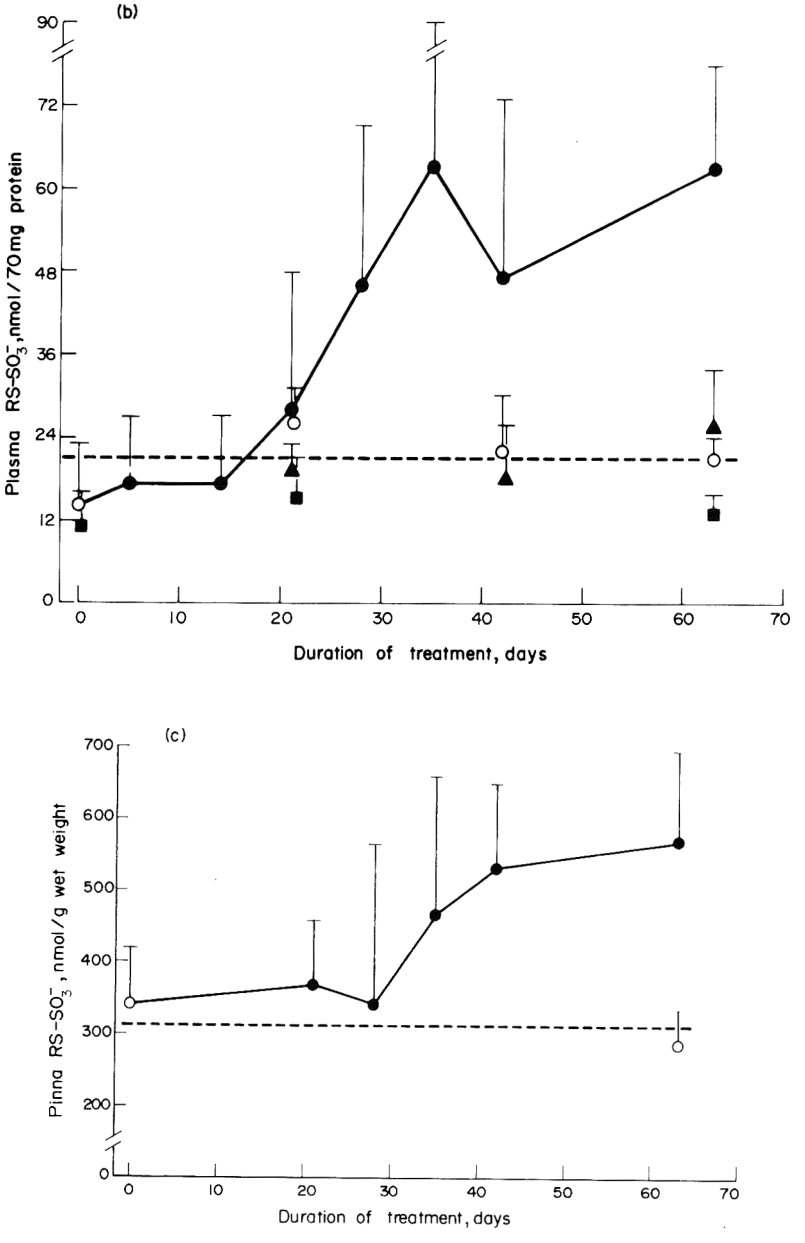

Fig. 2. *S*-Sulphonate concentrations in the aorta (a) plasma (b) and pinna (c) of sulphite-oxidase-deficient rats (NP/W, ●) and normal rats (NP, ○; PLC, ■; NP/W/Mo, ▲). Values are means ± 1 SD from determinations for approximately 6–12 rats in the sulphite-oxidase-deficient group and at least three rats in each of the control groups. The dotted line represents the mean concentration in the NP group over the entire experimental period.

t_{63}) plasma SO_3^{--} concentrations ranged from 0 to 60 nmol/ml and averaged 18 nmol/ml. No SO_3^{--} was detected in the plasma of six rats tested at t_{21} when the mean sulphite-oxidase activity was about 2·5 times greater than during the steady-state period.

The formation of RS–SO_3^- in the distal portion of the pinna of the ear was observed in sulphite-oxidase-deficient rats. Since this part of the ear consists predominantly of skin, we have assumed that RS–SO_3^- is associated with that tissue. This finding is consistent with RS–SO_3^- formation, as a function of SO_3^{--} exposure, in other elastic tissues such as the pulmonary lobes of the lung (Gunnison & Farruggella, 1979), the trachea (Gunnison *et al.* 1981), and, of course, the aorta. *S*-Sulphonate concentration in the pinna was not as responsive to decreasing sulphite-oxidase activity as was aorta and plasma RS–SO_3^- (Fig. 2c). Significant elevation above background levels was not observed in the NP/W group until day 35 of treatment and the maximum mean concentration attained (at t_{63}) was less than twice the mean background concentration.

Urinary RS–SO_3^- and $S_2O_3^{--}$ concentrations (nmol/mg creatinine), as a function of time of treat-

ment with the high-W/low-Mo regimen, are shown in Figs 3a and 3b respectively. The $S_2O_3^{--}$ data (Fig. 3b) were collected from a single series of rats, while each $RS\text{–}SO_3^-$ data point in Fig. 3a represents the mean of determinations from at least two and usually three urine pools collected from separate series of rats.

Urinary $RS\text{–}SO_3^-$ metabolites could be separated on a Sephadex G-25 column into fractions of large and small molecular weight. The large-molecular-weight fraction was associated with protein and was excluded from the Sephadex beads; it comprised only approximately 5% of the total $RS\text{–}SO_3^-$ in a sample containing 105 nmol/mg creatinine. Most of the $RS\text{–}SO_3^-$ in the sample, which was eluted in a single peak following the protein fraction, probably consisted predominantly of $Cys\text{–}S\text{–}SO_3^-$ but may also have contained other small molecular weight $RS\text{–}SO_3^-$ molecules such as glutathione *S*-sulphonate.

Excretion of $S_2O_3^{--}$ and $RS\text{–}SO_3^-$ (Fig. 3) generally correlated inversely with hepatic sulphite-oxidase activity, resulting in a similar temporal pattern of metabolite concentration, as was observed with tissue $RS\text{–}SO_3^-$. Only small amounts of these metabolites were excreted into the urine until t_{21} when sulphite-oxidase activity had declined to a specific activity of approximately 0·1 (2·6% of adult female activity). At this time a considerable increase in urinary $S_2O_3^{--}$ and $RS\text{–}SO_3^-$ was observed. Since normal background, or t_0, levels of $S_2O_3^{--}$ and $RS\text{–}SO_3^-$ are essentially zero, the amounts excreted at t_{21} were highly significant and a valid indication of decreased sulphite-oxidase activity. In addition, some, but not all, of the t_{14} urine samples contained easily detectable concentrations of $S_2O_3^{--}$ and $RS\text{–}SO_3^-$. The sensitivity of both of these analytical methods, as used in the experiments reported here, was approximately 3–5 nmol/mg creatinine assuming conditions of 100 and 50% analytical efficiency for $RS\text{–}SO_3^-$ and $S_2O_3^{--}$, respectively, and arbitrarily assigning a sample absorbance 0·02 optical density units greater than the matched blank as the minimum reliable response.

Urine was collected over only a portion of the time of steady state sulphite-oxidase activity, i.e. from t_{28}

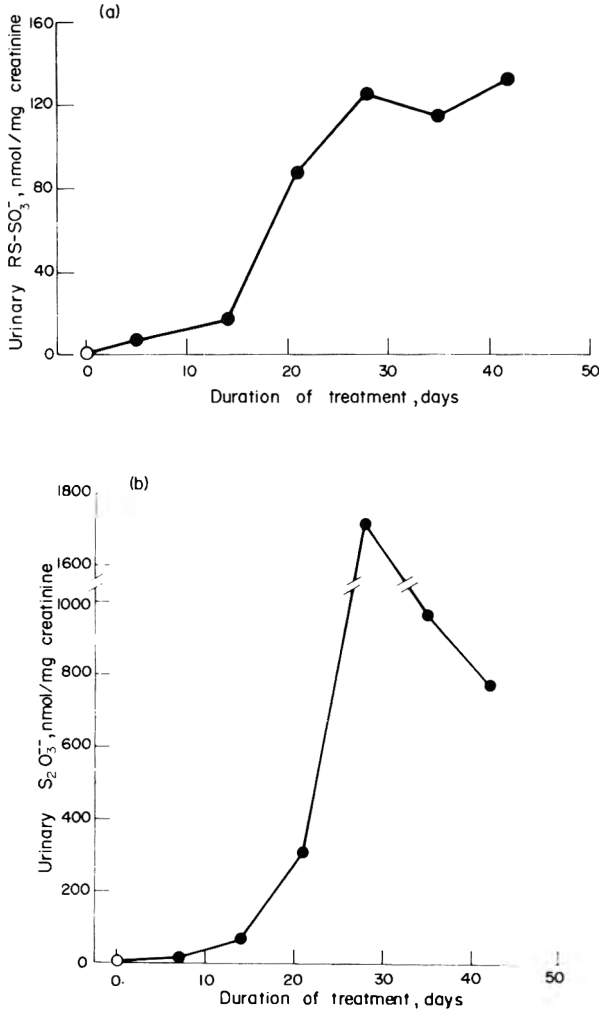

Fig. 3. Urinary *S*-sulphonate (a) and urinary thiosulphate (b) concentrations (nmol/mg creatinine) in sulphite-oxidase-deficient (NP/W, ●) and normal (NP, ○) rats. Values in (a) are means of determinations on two or three urine pools collected from separate series of rats; those in (b) are derived from a single series of rats.

to t_{42} inclusive. During this period the mean excretion of $RS–SO_3^-$ appeared to remain reasonably consistent, while excretion of $S_2O_3^{--}$ rose sharply at t_{28} and then declined. This irregularity of the $S_2O_3^{--}$ data presumably would have been significantly lessened had urine samples from several series of rats been analysed, as was the case for urinary $RS–SO_3^-$.

Although it appears from inspection of Figs 3a and 3b that the maximum concentrations of $S_2O_3^{--}$ excreted in the urine were approximately an order of magnitude greater than the corresponding $RS–SO_3^-$ concentrations, this difference may have been exaggerated by a somewhat biased selection of samples for analysis. Since SO_3^{--} gives a positive interference in the $RS–SO_3^-$ analytical procedure, urine samples that had high SO_3^{--} concentrations were excluded from $RS–SO_3^-$ analyses, while a similar screening procedure was not used prior to $S_2O_3^{--}$ analyses. A more accurate indication of the molar fraction of excreted $RS–SO_3^-$ and $S_2O_3^{--}$ can be obtained by direct comparison of four urine pools that were common to both analyses. In these, the molar fraction ($RS–SO_3^-/SO_2O_3^{--}$) was approximately 0·5 at t_{14} and decreased to 0·3 at t_{21} and to 0·2 at both t_{35} and t_{42}.

In most daily urine samples, SO_3^{--} was either not detected or was present in very low concentration (less than 10 nmol/mg creatinine), but some concentrations up to 2000 nmol/mg creatinine were encountered. The variability observed in excreted SO_3^{--} and metabolites of SO_3^{--} at similar t-times was greater among urine samples collected from different series of rats than among urine samples collected from the same series. In addition, much of the large individual variation in tissue $RS–SO_3^-$ metabolites (note dispersion in Fig. 2) could be traced to this 'interseries' variation. Sufficient data were not collected from individual series to allow precise correlation of sulphite-oxidase activity and metabolite production within each series; presumably, this would have reduced the variation observed.

Nevertheless, the curves illustrated in Figs 1, 2 and 3 should provide a reasonably accurate representation of the mean of the population, since, with the exception of urinary $S_2O_3^{--}$ data, rats from at least two and usually three or four series were represented in the construction of each one.

Metabolism of exogenous SO_3^{--}

Exogenous SO_3^{--} was administered to normal and sulphite-oxidase-deficient rats (1% of normal activity) by gastric intubation, and the resulting plasma SO_3^{--} concentrations were plotted with respect to time following intubation. The integrals of these curves, which were considered to be an expression of the effective systemic dose, are shown in Fig. 4 as a function of the logarithm of the intubated dose. As expected, much larger doses of intubated SO_3^{--} were required in normal rats than in sulphite oxidase-deficient rats to produce equivalent systemic doses of SO_3^{--}. Since there is no reason to suspect that pathways of SO_3^{--} elimination other than oxidation to sulphate are altered in sulphite-oxidase-deficient rats, the difference in the relationship of intubated and systemic doses between normal and deficient rats can be attributed to the activity of sulphite oxidase.

In normal rats, the plasma SO_3^{--} concentration following intubation generally showed a peak at less than 10 min, followed first by a rapid and then by a more gradual decline, which approached zero in less than 3 hr even at the highest intubated doses shown in Fig. 4. In sulphite-oxidase-deficient rats, the time course of plasma SO_3^{--} showed a more gradual rise to, and decline from, peak concentrations. Depending upon dose, peak concentrations in both deficient and normal rats ranged from approximately 100 to 1000 nmol/ml.

Figure 4 shows that, in the region of relatively low systemic SO_3^{--} doses (i.e. up to 10,000 nmol/ml × min), only approximately 10–20 times more gastric SO_3^{--} was required in normal rats than in deficient rats to produce equivalent systemic SO_3^{--} doses, in spite of the fact that normal rats contain approximately 100 times more enzyme, as determined by *in vitro* assay. This relationship is consistent with the theory that SO_3^{--} oxidation in the normal rat is not limited by the amount of sulphite oxidase in the mitochondria, but by the rate of diffusion of SO_3^{--} into the mitochondria (Oshino & Chance, 1975).

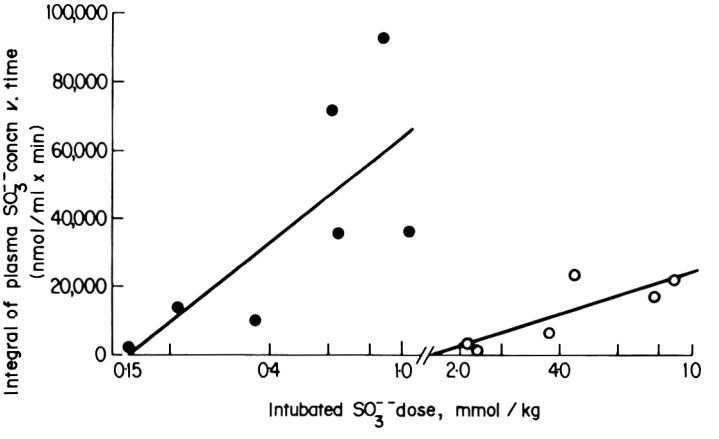

Fig. 4. The integral of plasma sulphite (SO_3^{--}) concentration versus time following gastric intubation of SO_3^{--} in normal (NP, ○) and sulphite-oxidase-deficient (NP/W, ●) rats, expressed as a function of the logarithm of the intubated dose. Curves are fitted to data points by the method of least squares for linear regression of the integral on the logarithm of the intubated dose.

According to this theory, at low levels of intubated SO_3^{--} the amount of sulphite oxidase in the mitochondria could be significantly decreased without proportionately affecting the rate of SO_3^{--} oxidation. Obviously, however, as sulphite oxidase continues to be depleted, it does at some point become critical to *in vivo* function; this is demonstrated in the sulphite-oxidase-deficient rats.

In Fig. 4, the relatively shallow slope of the integral *v.* log dose curve for normal rats suggests that over this range of intubated doses (up to approximately 8 mmol/kg) there was ample sulphite oxidase to maintain a relatively low level of systemic SO_3^{--}. However, when the dose range was extended to 12 mmol/kg, both of the treated animals died within 40 min from acute SO_3^{--} toxicity. Plasma SO_3^{--} in these rats rose dramatically after intubation (up to 3000 nmol/ml) and remained high until death, indicating that their sulphite-oxidase capacity had been overwhelmed. Paradoxically, the slower absorption of SO_3^{--} from the gut of sulphite-oxidase-deficient rats, a consequence of the lower doses administered, resulted in a balance between SO_3^{--} absorption and elimination, which permitted reasonably high and prolonged plasma SO_3^{--} concentrations; this is reflected in the magnitude of their integrals.

DISCUSSION

As stated at the outset, our primary reason for characterizing SO_3^{--} metabolism in sulphite-oxidase-deficient rats was to provide background data for their ultimate utilization in the evaluation of potential human SO_3^{--} toxicity. We have demonstrated the feasibility of a sulphite-oxidase-deficient rat model with mean steady-state hepatic activity about 1% of that of normal adults. Assuming that sulphite-oxidase activity in the normal rat is ten times greater than that in man (Johnson & Rajagopalan, 1976a,b) this rat model is metabolically equivalent to humans containing only 10% of their usual enzyme activity and thus should constitute a more sensitive animal model than the normal rat for evaluation of possible human toxicity.

In the sulphite-oxidase-deficient rat model described in this paper, high systemic doses of SO_3^{--} were produced by the gastric intubation of relatively small amounts of SO_3^{--}. In addition, significant systemic exposure resulted from the catabolism of endogenous sulphur-containing amino acids. Furthermore, it was shown that steady-state sulphite-oxidase levels could be manipulated by regulation of the W/Mo intake ratio, thus providing a means of altering the systemic exposure from a given sulphur-containing amino acid or SO_3^{--} input. We believe, therefore, that this model offers flexibility in the regulation of systemic SO_3^{--} exposures and that these can be adequately monitored and characterized by methods described in this paper.

The results of our experiments clearly demonstrated an inverse relationship between sulphite-oxidase activity and the formation of the SO_3^{--} metabolites, $RS\text{–}SO_3^-$ and $S_2O_3^{--}$. Thus, an inhibition or 'narrowing' of the usual oxidative metabolic pathway for SO_3^{--} resulted in its greater availability for passage along other metabolic routes, and presumably also enhanced the chance of its participation in potentially toxic reactions. Thiosulphate and $RS\text{–}SO_3^-$ metabolites appeared in the urine prior to the significant accumulation of $RS\text{–}SO_3^-$ in the tissues. These urinary metabolites were first detected when the sulphite-oxidase level was reduced to about 7% of the normal adult level (at t_{14}). A further reduction in enzyme activity to 2·6% of the norm (at t_{21}) resulted in their excretion in relatively large amounts. In the plasma, statistically significant accumulation of $RS\text{–}SO_3^-$ was not apparent until sulphite-oxidase activity was approximately 1% of the normal adult level (at t_{28}), although the recognition of low-level $RS\text{–}SO_3^-$ accumulation was made difficult by the variation observed in control animals. The same problem existed with the aorta and ear skin, but not with urine since endogenous values in the urine of control rats were essentially zero for both $S_2O_3^{--}$ and $RS\text{–}SO_3^-$. Thus, urinary metabolites were the most sensitive indicators of decreased sulphite-oxidase activity and, moreover, the collection of urine samples was simple, convenient and did not require the use of invasive or destructive techniques. On the other hand, tissue $RS\text{–}SO_3^-$ metabolites, because of their relative *in vivo* stability (Gunnison & Farruggella, 1979; Gunnison & Palmes, 1978) can provide some indication of recent exposure history which may not be apparent from analysis of a single 16-hr urine sample.

Of the chemical indicators of decreased sulphite-oxidase activity investigated in these experiments, free SO_3^{--} in plasma and/or urine is the least sensitive. This is apparent from the usual absence of SO_3^{--} from urine samples containing easily measurable quantities of $S_2O_3^{--}$ and $RS\text{–}SO_3^-$ and the undetectable or low concentrations of plasma SO_3^{--} in rats whose tissues contained relatively high concentrations of $RS\text{–}SO_3^-$ metabolites. The half-life of plasma SO_3^{--} in normal rats is very short (Gunnison & Palmes, 1976) and apparently even in the sulphite-oxidase-deficient rats of this model, it is sufficiently short to prevent significant accumulation.

The accumulation of SO_3^{--} in urine is inhibited by its instability in that medium, as observed in our laboratory and also as reported by other investigators (Irreverre, Mudd, Heizer & Laster, 1967; Kutter & Humbel, 1969). Several lines of evidence point to the presence in the urine of a substance, probably an oxidizing agent, that destroys SO_3^{--}. After sufficient SO_3^{--} has been excreted into the urine to deplete it of this presumed oxidant, the stability of urinary SO_3^{--} improves. The existence of an endogenous urinary oxidant provides a credible explanation for the poor analytical recovery of $RS\text{–}SO_3^-$ added to urine not treated with XAD. Since the accurate estimation of $RS\text{–}SO_3^-$ depends on the determination of SO_3^{--} generated by cyanolysis during the analytical procedure, destruction of the cyanolysed SO_3^{--} by an endogenous oxidant in urine would result in a low estimate of $RS\text{–}SO_3^-$. Apparently XAD resin removes most of this endogenous oxidant since treatment of urine with the resin markedly improves analytical recovery. The same presumed endogenous urinary oxidant is probably also responsible for the oxidation of $S_2O_3^{--}$ added to urine collected from control rats (Table 2). Thiosulphate added to urine collected from sulphite-oxidase-deficient rats, however, was much

more stable. Apparently SO_3^{--} excreted into the urine of these rats saturated (or at least greatly diminished) the capacity of the endogenous urinary oxidant, thus improving the stability of $S_2O_3^{--}$ added to the urine sample.

Three cases of human sulphite-oxidase-deficiency disease have been documented in the literature (Duran, Korteland, Beemer, van der Heiden, de Bree, Brink & Wadman, 1979; Irreverre *et al.* 1967; Shih, Abroms, Johnson, Carney, Mandell, Robb, Cloherty & Rajagopalan, 1977). Each of these was characterized metabolically by the urinary excretion of large amounts of Cys–S–SO_3^- and $S_2O_3^{--}$ and a decreased amount of sulphate. The concentrations of Cys–S–SO_3^- reported in the urine of the human cases are approximately an order of magnitude greater than the urinary RS–SO_3^- observed in the rat model at steady-state enzyme activity, while $S_2O_3^{--}$ concentrations generally ranged up to levels five times greater in the human cases. Also, free SO_3^{--} was consistently present at a slightly higher molar concentration than $S_2O_3^{--}$ in urine from the sulphite-oxidase-deficient humans. Thus, if one assumes an equivalent dietary intake of sulphur-containing amino acids, it would appear that sulphite-oxidase activity is lower in the human cases than in the rat model. With the exceptions of cysteine- and methionine-loading experiments carried out in the cases of human sulphite-oxidase-deficiency disease and the studies reported here, the quantitative relationships between sulphite-oxidase activity, dietary sulphur-containing amino acid intake and SO_3^{--} metabolism have not been studied in a controlled experimental situation. Once these relationships are better understood, information on dietary intake of sulphur-containing amino acids and on excretion of SO_3^{--} and its metabolites should enable the accurate prediction of the sulphite-oxidase activity in man or animals.

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A SULPHITE-OXIDASE-DEFICIENT RAT MODEL: SUBCHRONIC TOXICOLOGY

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Abstract—Toxicity resulting from exposure to sulphite originating both endogenously and exogenously was investigated in normal rats and in rats made sulphite-oxidase-deficient by molybdenum deficiency abetted by administration of tungstate. The sulphite-oxidase-deficient rats were outwardly as healthy as controls and exhibited normal weight gain and maintenance over the 9-wk test period. The systemic sulphite exposures of normal and deficient rats resulting from various sulphite treatments could be compared by determining the concentrations of tissue *S*-sulphonate ($RS-SO_3^-$) metabolites formed. In general, relatively low intakes of exogenous sulphite (0–3·5 mmol/kg/day) by sulphite-oxidase-deficient rats produced systemic sulphite exposures equivalent to those produced by the ingestion by normal rats of highly sulphited diets (intakes of 13–25 mmol/kg/day). The advantages of the sulphite-oxidase-deficient rat compared to the normal rat as a model for human exposure are discussed. Using these two animal models, it was demonstrated that anaemia and thiamine deficiency, which have been produced previously in sulphite-feeding studies, result solely from the action of high concentrations of sulphite in the diet and/or gut and are not attributable to systemic sulphite exposure. Likewise, prothrombin time and erythrocyte concentrations of glutathione were not affected by high systemic sulphite concentrations in these experiments.

A 4/149 incidence of mammary adenocarcinoma was observed in sulphite-oxidase-deficient rats, all in rats aged less than 5 months, compared to 0/143 observed in age-matched rats with normal sulphite oxidase. Although this result was not statistically significant, the rarity of spontaneous tumours of this type among rats of this age suggests that these carcinomas may, in fact, have been treatment related. If indicated, further investigation will be undertaken to determine the role of sulphite-oxidase-deficiency, sulphite and/or tungstate, as well as other elements of the model, in the aetiology of these tumours.

INTRODUCTION

Sulphite and bisulphite salts (referred to throughout as sulphite, SO_3^{--}) have been utilized as food additives for many years because of their antioxidant and bacteriostatic properties. Sulphur dioxide (SO_2), the anhydride of SO_3^{--}, has historically been present as a pollutant in community and industrial atmospheres. Sulphite uptake from these sources augments a larger SO_3^{--} burden generated endogenously by the catabolism of sulphur-containing amino acids (Institute of Food Technologists and Committee on Public Information, 1976). Regardless of the source, *in vivo* SO_3^{--} is oxidized to sulphate ion, a reaction catalysed by the enzyme sulphite oxidase. We have previously suggested that the high efficiency of this enzyme can explain the relatively low mammalian toxicity of SO_3^{--} compared with its potentially damaging reactions demonstrated by *in vitro* experiments (Gunnison & Palmes, 1976).

Research on the toxicity of ingested SO_3^{--} has been complicated by reactions of SO_3^{--} with constituents of the diet prior to ingestion. Sulphite can substantially decrease the thiamine content of diets that are not properly stored (Fitzhugh, Knudsen & Nelson, 1946) and can also react with unsaturated dietary fats (Institute of Food Technologists and Committee on Public Information, 1976). Bhagat & Lockett (1964) have demonstrated, in addition to thiamine deficiency, toxic signs such as diarrhoea and stunting of growth in rats fed sulphited diets that had aged at room temperature. These signs were not observed by other investigators when thiamine-supplemented sulphited diets were stored in the frozen state prior to administration.

In several studies designed to assess the primary toxicity of SO_3^{--} *in vivo*, the possibility of indirect toxicity resulting from the reactions of SO_3^{--} in the diet was eliminated by administration of SO_3^{--} in the drinking-water, or by the thiamine-supplementation and proper storage of sulphited diets (Cluzan, Causeret & Hugot, 1965; Lanteaume, Ramel, Girard, Jaulmes, Gasq & Ranau, 1965; Lockett & Natoff, 1960; Til, Feron & De Groot, 1972). The Joint FAO/WHO Expert Committee on Food Additives (1974) used the most comprehensive of these studies (Til *et al.* 1972) as the basis for the establishment of an acceptable daily intake (ADI) of 0·70 mg SO_2/kg. Til *et al.* (1972) fed three generations of rats on thiamine-supplemented diets containing levels of sodium metabisulphite ranging from 0 to 2%. They found occult blood in the faeces of a small percentage of rats ingesting 0·25% dietary metabisulphite and an increased frequency and severity at higher levels, presumably due to irritation of the gastro-intestinal tract. Pathological changes in the stomach mucosa were observed with 1 and 2% dietary metabisulphite and some slight growth retardation with 2%. In shorter-term exposures lasting up to 8 wk, 6% metabisulphite in the diet led to severe growth retardation and

anaemia, as well as to decreases in food consumption and efficiency. All these effects, in short- and long-term exposures, were reversible.

Most, and perhaps all, of the SO_3^{--}-induced toxic changes detected in experiments to date can be attributed to high dietary concentrations of SO_3^{--}, which destroy the activity of dietary thiamine and perhaps other nutrients and/or produce pathological changes in the gut after ingestion. In no case can toxicity be attributed with certainty to systemic SO_3^{--}; indeed, the effective systemic exposure during and following ingestion has not been addressed by most previous investigators.

It is apparent from more recent work in this and other laboratories, that the most critical factor in the regulation of systemic SO_3^{--} concentration is sulphite-oxidase activity (Gunnison, Farruggella, Chiang, Dulak, Zaccardi & Birkner, 1981; Gunnison & Palmes, 1976 & 1978; Shih, Abroms, Johnson, Carney, Mandell, Robb, Cloherty & Rajagopalan, 1977). Since the rat is well endowed with sulphite oxidase, relatively large doses of SO_3^{--} administered intragastrically to this species result in surprisingly low systemic levels (Gunnison *et al.* 1981). On the other hand, primates, including man, have relatively low sulphite-oxidase activity (Johnson & Rajagopalan, 1976) and, therefore, identical doses of ingested SO_3^{--} should produce a considerably greater systemic load in man than in the rat. One of the goals of the research reported here is to demonstrate the modulating effect of sulphite oxidase on the effective systemic exposure resulting from ingested SO_3^{--}. To accomplish this we have used sulphite-oxidase-deficient as well as normal rats. The protocol for development of sulphite-oxidase-deficient rats was introduced by Rajagopalan and co-workers (Johnson, Rajagopalan & Cohen, 1974) and was recently adopted by us in the production of a group of sulphite-oxidase-deficient rats which we subsequently characterized metabolically (Gunnison *et al.* 1981). At steady state, these rats possess an estimated one tenth of the hepatic sulphite-oxidase concentration of normal humans and in this respect may be the analogue of asymptomatic, sulphite-oxidase-deficient humans. There has been speculation about such a human population (Hickey, Clelland, Bowers & Boyce, 1976), but no confirmation of its existence.

The primary thrust of the experiments reported here is the investigation, in sulphite-oxidase-deficient rats, of possible toxicity resulting from subchronic systemic SO_3^{--} exposure. The major experiment of this study was designed to investigate the teratogenicity of SO_3^{--}. In addition, however, certain biochemical and physiological parameters were monitored for the detection of other SO_3^{--}-related toxic changes. These results are reported in this article while results of the teratological aspects will be reported in a separate publication.

EXPERIMENTAL

Design. Virgin female Wistar rats weighing approximately 200–225 g and purchased from Marland Breeding Farms, Hewitt, NJ, were assigned to one of seven different groups (Table 1). Four of these were designated as control groups for several variables and possessed normal levels of hepatic sulphite-oxidase activity. The remaining three were designated as treatment groups and were made deficient in sulphite-oxidase activity as described previously (Gunnison *et al.* 1981), the steady-state hepatic enzyme levels being approximately 1% that of untreated adult rats. This was achieved by maintenance on a low-molybdenum (Mo) normal-protein diet (no. 902487, ICN Nutritional Biochemicals, Cleveland, OH), with concurrent addition to the drinking-water of sodium tungstate at the level of 200 ppm tungsten (W) for a period of 28 days, and thereafter at 100–120 ppm W for an additional period of approximately 35 days (standard 'high-W/low-Mo' regimen). The drinking-water of two of these groups was also supplemented with sodium metabisulphite to yield a concentration of 25 and 50 mM-SO_3^{--} respectively, beginning on day 21 (t_{21}) of tungsten administration. These three groups were designated NP/W, NP/W/$SO_3^{--}{}_{(25)}$ and NP/W/$SO_3^{--}{}_{(50)}$ as shown in Table 1.

The four control groups included one diet control group (PLC) which received Purina Rodent Lab Chow (PLC; Ralston Purina Co., St. Louis, MO) and tap-water. A second diet control group (NP) was given the normal-protein (NP) diet and glass-distilled water to assess the effects of the low-Mo diet. The remaining two control groups were used to assess W toxicity other than that resulting as a consequence of

Table 1. *Regimens and sulphite-oxidase status of experimental groups*

Experimental group	Treatment or control	Sulphite-oxidase status	Diet	Drinking-water supplementation
PLC	C	Normal	Purina Rodent Lab Chow	None
NP	C	Normal	Normal protein	None
NP/W/$SO_4^{--}{}_{(25)}$	C	Normal	Normal protein	W (100–200 ppm) Mo (4 ppm) Na_2SO_4 (12·5 mM)
NP/W/Mo/$SO_4^{--}{}_{(50)}$	C	Normal	Normal protein	W (100–200 ppm) Mo (4 ppm) Na_2SO_4 (25 mM)
NP/W	T	Deficient	Normal protein	W (100–200 ppm)
NP/W/$SO_3^{--}{}_{(25)}$	T	Deficient	Normal protein	W (100–200 ppm) $Na_2S_2O_5$ (≡ 25 mM-SO_3^{--}
NP/W/$SO_3^{--}{}_{(50)}$	T	Deficient	Normal protein	W (100–200 ppm) $Na_2S_2O_5$(≡ 50 mM-SO_3^{--})

displacement of Mo. These groups were initially given drinking-water containing sodium tungstate at 200 ppm W and sodium molybdate at 4 ppm Mo. After 28 days the W concentration was decreased, as in the treatment groups, to 100–120 ppm W, Mo concentration remained at 4 ppm and sodium sulphate was added to the drinking-water (on t_{21}) to provide concentrations of sodium equimolar to the 25 and 50 mM SO_3^{--}-treated groups. The animals in these groups were then matched for weight and water consumption with their respective SO_3^{--}-treated counterparts and pair watered to control for the SO_3^{--}-induced decrease in fluid consumption. These two groups were designated NP/W/Mo/$SO_4^{--}{}_{(25)}$ and NP/W/Mo/$SO_4^{--}{}_{(50)}$ as shown in Table 1 and are referred to collectively as the Mo controls.

All drinking-solutions were made up in glass-distilled water. Fresh SO_3^{--}-supplemented solutions were prepared daily to minimize decreases in SO_3^{--} concentration due to autoxidation; these were determined to be negligible over a 24-hr period. Fluid consumption was measured daily and diet consumption was measured on alternate days for all rats except those in the PLC, NP and NP/W groups where subgroups of at least ten animals were used. Consumption was not corrected for wastage although rats observed to waste large amounts of food or water consistently were excluded from the group averages.

Hepatic sulphite-oxidase activity in the various groups was monitored by intermittent killing and assay according to the method of Johnson *et al.* (1974) and Kessler & Rajagopalan (1972), as previously described (Gunnison *et al.* 1981). At the beginning of wk 7 (t_{43}), all rats were mated with normal males maintained on a diet of PLC and tap-water. Male rats were brought to the cage of the female on test so that the dietary treatment of the female was not interrupted. All groups contained approximately 20 pregnant rats except the NP/W/$SO_3^{--}{}_{(25)}$ and NP/W/Mo/$SO_4^{--}{}_{(25)}$ groups which contained 14 and 11 respectively. Pregnant animals were killed on day 21 of gestation (also designated t_{63}), with the day that a vaginal plug was found considered as day 0. Rats that were paired but not pregnant (10–20% of the total in each group) were killed at the same time.

A second series of experiments of shorter duration was conducted with adult female rats possessing normal levels of sulphite-oxidase activity. The rats in these experiments were not mated. Four groups of rats were given tap-water and NP powdered diet incorporated with either 0, 1, 2 or 6% powdered sodium metabisulphite. Diets containing metabisulphite, but not the control diet, were supplemented with 50 ppm thiamine. Uniform distribution was attained using a rotary mixer. The diets were prepared every 4 or 5 days and stored at −10°C, and a fresh portion was given to the rats each day. Disappearance of SO_3^{--} was negligible during storage at −10°C and minimal (i.e. up to 8% in the 1% diet) over a 24-hr period at room temperature. Corrections for losses at room temperature were made in the calculation of SO_3^{--} intake.

Biochemical and physiological measurements

Haemoglobin (*Hb*). Whole blood collected from the tail in a 20-μl capillary tube without anticoagulant was mixed with 5 ml of a modified Drabkin's reagent containing 2·60 mM-citric acid, 0·61 mM-potassium ferricyanide, 0·77 mM-potassium cyanide, 8·25 mM-tris-(hydroxymethyl)aminomethane and 10% (v/v) ethylene glycol. The haemoglobin content of this mixture was determined as cyanmethaemoglobin by comparison of the optical density with that of standard cyanmethaemoglobin (Simmons, 1976).

Red blood cell reduced glutathione (*RBC-GSH*). Reduced glutathione in RBCs was determined by a method given by Beutler (1975) using 40 μl of whole blood collected as described for Hb determination and assuming all GSH to be derived from the RBCs. Blood was always collected between 08.30 and 12.30 hr, alternating among rats of different groups to randomize the effect of the circadian variation of GSH concentration.

Prothrombin time. The one-stage prothrombin time test is sensitive to reduced concentrations of Factors II, V, VII and X and thus assays for vitamin K function. Citrated plasma for the test was collected by heart puncture, 4·5 ml blood being drawn into a syringe containing 0·5 ml 3·8% sodium citrate. The plasma was separated, quick frozen at −90°C and stored at that temperature until the time of analysis. Plasma samples were thawed at 37°C and tested immediately in duplicate in the one-stage prothrombin time test of Quick (Simmons, 1976) using rabbit-brain thromboplastin with calcium obtained from Ortho Diagnostics, Raritan, NJ. An Embdeco Prothrombin Timer was used to determine the time of coagulation. Normal and abnormal human plasma standards were tested as controls at the time of sample analysis. The quick freezing and thawing technique did not affect the prothrombin times of these standards.

Haematocrit (*Hc*). Haematocrit determinations were made with tail blood collected in standard heparinized capillary hematocrit tubes.

Hepatic thiamine. When the rats were killed, pieces of liver were removed and frozen at −90°C until assay. Hepatic thiamine content (μg/g wet weight) was determined by the fluorometric method of the Association of Official Analytical Chemists (1975).

Tissue S-sulphonate (*RS-SO_3^-*) *analyses.* *S*-Sulphonate concentrations were determined in the aorta, distal portion of the pinna of the ear and plasma as described previously (Gunnison *et al.* 1981). Tissues were removed at death and stored at −90°C until analysed. Plasma was obtained from either citrated or heparinized blood collected by heart puncture. Plasma-protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Sulphite-oxidase activity

Hepatic sulphite-oxidase activity in rats of the treatment and control groups is illustrated as a function of time in Fig. 1. It was previously demonstrated that sulphite-oxidase activity decreases exponentially over the first 28 days in rats treated with the standard 'high-W/low-Mo' regimen and thereafter maintains an approximately steady-state level of about 1% of the normal adult activity. The rats in the treatment groups represented in Fig. 1 include some of those

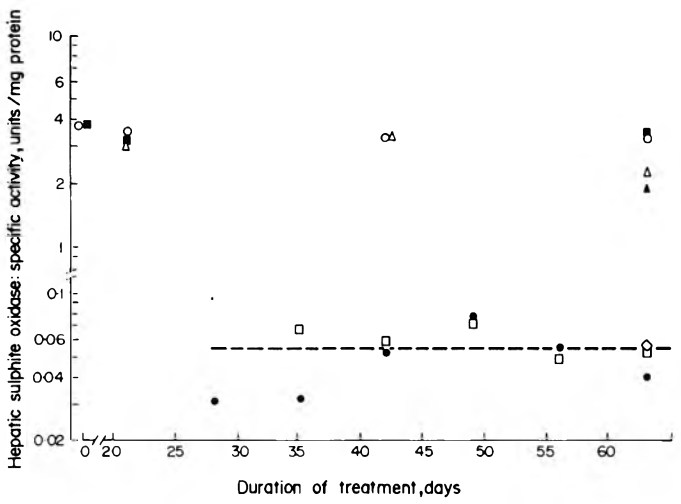

Fig. 1. Hepatic sulphite-oxidase activity as a function of time in groups of rats maintained on several experimental regimens (identified in Table 1): PLC (■), NP (○), NP/W/$SO_4^{--}{}_{(25)}$ (△), NP/W/$SO_4^{--}{}_{(50)}$ (▲), NP/W (●), NP/W/$SO_3^{--}{}_{(25)}$ (◇), NP/W/$SO_3^{--}{}_{(50)}$ (□). Values are means of measurements from 3–9 rats and the dotted line is the mean of data points for all sulphite-oxidase-deficient rats. These data include some that have previously been reported (Gunnison *et al.* 1981).

used in the previous metabolism study (Gunnison *et al.* 1981) as well as those used in this study exclusively. The sulphite-oxidase-deficient rats receiving exogenous SO_3^{--} exhibited activity similar to that of the NP/W group, and the mean steady-state activity of all treatment groups was about 1–2% of normal adult levels. The sulphite-oxidase activity of the control groups remained in the range of normal adults throughout the experimental period (using the PLC group as a standard) although the activity of the Mo controls was slightly depressed at t_{63}.

General health of animals

Approximately 10–15% of the rats in the NP group showed signs of diarrhoea, usually within 3 wk of the beginning of this regimen. In most cases the diarrhoea was not severe and did not persist in spite of continued administration of the diet. The percentage of rats showing signs of diarrhoea increased to approximately 20–25% in the NP/W group and 30% in the NP/W/Mo group, although these percentages varied among different shipments of rats. As with rats in the NP group, diarrhoea usually lasted only a few days and disappeared spontaneously. In a few cases in all groups, however, the diarrhoea became progressively worse and these animals were removed from the experiment. Tests were run on some of the worst cases for pathogenic enteric protozoa, helminths and pathogenic species of Salmonella, Pseudomonas and Citrobacter (AnMed Labs, New Hyde Park, NY). The results were consistently negative for all of these organisms. Further, the signs of diarrhoea cleared up completely within 1 or 2 days when affected rats were transferred to a diet of PLC. In addition, no signs of diarrhoea were observed in the PLC experimental group. On the basis of these findings, it appeared that the diarrhoea could be attributed to the NP diet and was apparently exacerbated by the administration of W and possibly also by Mo. No other overt signs of toxicity were apparent in any of the experimental groups.

The diarrhoea that developed during the course of these studies was an inconvenience but not a serious obstacle to the production of sulphite-oxidase-deficient rats. Rajagopalan and co-workers in their studies with sulphite oxidase-deficient rats, used the NP diet and did not report problems with diarrhoea (Johnson *et al.* 1974). We have since found that diarrhoea, if it is a problem, can be eliminated without compromising the model by substitution of the NP diet with a similar low-MO diet containing 5% fibre (AIN-76 semi-purified diet, ICN Nutritional Biochemicals).

Food and water consumption

Mean water consumption by the NP-diet control rats was 27 ml/rat/day prior to mating (t_0-t_{42}) and 37 ml/rat/day during gestation. Among the treatment groups, addition of W alone did not alter fluid consumption, but further addition of 25 mM- and 50 mM-SO_3^{--} caused decreases of approximately 26 and 35%, respectively, prior to mating and 14 and 35% during gestation. All these changes were statistically significant except the 14% reduction. The concentration of W in the NP/W/SO_3^{--} groups was increased to offset the reduction in the volume consumed. The Mo control groups which were pair watered with their respective SO_3^{--} treatment groups showed some agitation as a result of their fluid restriction.

Food consumption in the NP-diet control group in the periods prior to mating and during gestation averaged 16·6 and 21·2 g/rat/day respectively. The treatment groups generally consumed slightly smaller amounts of food (0–6% decrease) with no apparent SO_3^{--}-related trend. None of these differences were statistically significant. The average daily food consumption for the Mo control groups showed slightly greater decrements. Compared to the NP control

group, an average decrease of 7% was observed prior to mating and 12% during gestation. The latter difference was statistically significant ($P < 0.05$).

The PLC-diet control group consumed approximately 30% more food and water than the NP group. This difference in consumption was probably a result of the relatively high fibre and low protein content of the PLC diet compared to the NP diet.

In a pilot experiment, food and water consumption was measured in a group of eight sulphite-oxidase-deficient rats given 75 mM-SO_3^{--} in their drinking water (NP/W/$SO_3^{--}{}_{(75)}$). Fluid consumption was depressed by approximately 50% and food consumption by about 10% relative to NP controls. Weight gain in this group was also poor and additional experimentation with this treatment was not undertaken.

Body and organ weights

Table 2 summarizes the weight gain of rats in each group prior to mating (t_0–t_{42}) and during gestation, and also gives organ weights in terms of percentage of body weight at the end of the experimental period (approximately t_{63}). These data for each group were compared by Student's *t* test against the NP-diet control group. In general, there were no trends that could be correlated with SO_3^{--} treatment. In cases where there was statistically significant variation from the NP-diet control group, these deviations were usually greatest or occurred only in the Mo control groups. For example, Mo control groups showed decreases with a high degree of statistical significance in both body weight and liver weight (expressed as a percentage of body weight). These results may be attributable, at least in part, to the decrease in food consumption and the agitation due to pair watering in the Mo control groups (Oishi, Oishi & Hiraga, 1979), although a direct toxic effect of Mo is also a possible explanation. Levels of Mo higher than those used here have been observed to cause growth retardation in cattle and rats (Friberg, 1979).

Kidney weights (as a percentage of body weight) were significantly increased in the NP/W/$SO_3^{--}{}_{(50)}$ group, but not in the other two lower-dose treatment groups. In their multigeneration studies, Til *et al.* (1972) observed a similar change in the high-dose group (2% dietary sodium metabisulphite), but only in the F_2 generation and the weight increase was not accompanied by functional or histological alterations.

Biochemical and physiological measurements

In preliminary experiments in which rats were maintained on NP diet and water containing 400 ppm W plus 50 or 100 mM-SO_3^{--}, some animals showed traces of a dried, haem-like substance around the nostrils. This observation, together with a previous demonstration by Carmack, Moore & Balis (1950) of the reaction of SO_3^{--} with menadione, a synthetic vitamin K present in the NP diet, prompted us to investigate prothrombin times in our later, more detailed experiments. In addition, in these later experiments, we also measured Hb, Hc, RBC-GSH and hepatic thiamine. Table 3 summarizes the results of these measurements made on pregnant rats on day 21 of gestation (t_{63}). Data from the NP-diet control group were statistically compared by Student's *t* test against each of the other groups. There were no treatment-related trends in any of the parameters measured in spite of small but statistically significant differences between some group pairs. Also, with the exception of prothrombin times and hepatic thiamine concentration, the absolute differences observed between the two diet control groups (NP and PLC) were larger than those of any of the other pairs compared.

The Hb and Hc levels shown in Table 3 reflect a general anaemia of pregnancy. For comparison, in non-pregnant rats from the same experiment maintained on NP diet (i.e. only PLC group excluded), the mean values of Hb and Hc for all groups combined were 16·3 g/100 ml whole blood and 49%, respectively. There were no obvious differences among the non-pregnant rats of the various groups, although the numbers of animals in each group were too small to permit sensitive comparisons.

Failure to produce anaemia in W- and particularly W/SO_3^{--}-treated animals was somewhat surprising in view of previous experiments by Til *et al.* (1972). These authors produced severe anaemia by feeding young male Wistar rats a diet containing 6% sodium metabisulphite for 3 wk, slight anaemia by feeding 2% metabisulphite and no evidence of anaemia with a diet containing 1% metabisulphite. Results after 8 wk of treatment were essentially the same. We repeated this experiment for a 3-wk period by feeding four groups of rats on NP powdered diet incorporating 0.1, 2 and 6% powdered sodium metabisulphite.

Table 2. *Body and tissue weights in experimental groups of sulphite-oxidase-deficient and normal rats*

Experimental group†	Body-weight gain t_0–t_{42} (g)	Body-weight gain Gestation (g)	Body weight at t_{63} (g)	Organ weight at t_{63} (% body weight) Liver	Kidney	Spleen
PLC	61 ± 14*	152 ± 25	440 ± 34	3·36 ± 0·37	0·46 ± 0·04	0·158 ± 0·020
NP	70 ± 14	143 ± 20	440 ± 33	3·42 ± 0·31	0·48 ± 0·06	0·149 ± 0·020
NP/W/Mo/$SO_4^{--}{}_{(25)}$	54 ± 17**	119 ± 12**	401 ± 27**	2·89 ± 0·25**	0·47 ± 0·04	0·141 ± 0·034
NP/W/Mo/$SO_4^{--}{}_{(50)}$	50 ± 13***	123 ± 15**	389 ± 18***	3·08 ± 0·31**	0·51 ± 0·06	0·145 ± 0·018
NP/W	60 ± 14*	140 ± 21	429 ± 27	3·21 ± 0·33*	0·48 ± 0·05	0·162 ± 0·023
NP/W/$SO_3^{--}{}_{(25)}$	59 ± 13*	131 ± 19	421 ± 30	3·23 ± 0·14*	0·47 ± 0·04	0·154 ± 0·023
NP/W/$SO_3^{--}{}_{(50)}$	69 ± 19	147 ± 27	443 ± 54	3·51 ± 0·31	0·55 ± 0·06***	0·156 ± 0·024

† See Table 1 for description of experimental groups.

Values are means ± SD and those marked with asterisks differ significantly (Student's *t* test) from the value for the NP group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. *Biochemical and physiological parameters in experimental groups of sulphite-oxidase-deficient and normal pregnant rats on day 21 of gestation† following approximately 63 days of treatment*

Experimental group‡	Hb (g/100 ml blood)	Hc (%)	Hb/Hc	Prothrombin time (sec)	RBC-GSH (mg/100 ml RBC)	Hepatic thiamine (μg/g wet weight)
PLC	12·5 ± 0·8*** (21)	37·4 ± 2·3*** (20)	0·34 ± 0·01 (20)	13·1 ± 0·5 (7)	96·0 ± 9·5* (7)	11·2 ± 1·7 (3)
NP	13·5 ± 0·6 (20)	40·4 ± 2·4 (20)	0·33 ± 0·02 (20)	13·2 ± 0·9 (10)	73·9 ± 10·4 (9)	12·8 ± 0·4 (3)
NP/W/Mo/SO_4^{--} (25)	13·1 ± 0·9 (11)	39·5 ± 2·1 (11)	0·33 ± 0·01 (11)	12·8 ± 0·9 (6)	73·2 ± 8·2 (8)	15·3 ± 2·1 (3)
NP/W/Mo/SO_4^{--} (50)	13·6 ± 1·0 (17)	42·0 ± 2·1* (17)	0·32 ± 0·02 (17)	13·8 ± 0·2 (7)	67·6 ± 10·6 (9)	—
NP/W	12·9 ± 0·6** (17)	40·2 ± 1·9 (17)	0·32 ± 0·01 (17)	12·1 ± 0·5* (6)	80·7 ± 8·9 (13)	14·6 ± 1·0* (3)
NP/W/SO_3^{--} (25)	12·6 ± 0·8*** (14)	39·2 ± 2·0 (14)	0·32 ± 0·02 (14)	12·3 ± 0·6* (9)	75·6 ± 7·3 (11)	14·7 ± 2·7 (3)
NP/W/SO_3^{--} (50)	13·1 ± 1·0 (20)	40·8 ± 2·3 (21)	0·32 ± 0·02 (20)	13·8 ± 0·6 (11)	82·8 ± 12·4 (11)	12·1 ± 1·8 (3)

Hb = Haemoglobin Hc = Haematocrit RBC–GSH = Red blood cell, reduced glutathione

† Day of vaginal plug considered as day zero.
‡ See Table 1 for description of experimental groups.
Values are means ± 1 SD for the numbers of animals indicated in brackets and those marked with asterisks differ significantly (Student's *t* test) from the value for the NP group:
*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 4 summarizes Hb and Hc data, as well as plasma prothrombin times and hepatic thiamine concentrations, at the termination of the 3-wk feeding period. No effect on prothrombin time was observed in the 6% group. Our results for Hb concentration and Hc are similar both qualitatively and quantitatively to those observed by Til *et al.* (1972). Moreover, the Hb/Hc ratio (Table 4) which is usually very consistent (see Table 3) decreased significantly in the rats fed 6% metabisulphite. This reflects a greater decrease in Hb concentration than in Hc and, therefore, indicates that the anaemia observed cannot be fully explained by a change in blood volume or other factors that might merely decrease the concentration of RBCs in the tail blood. A similar value calculated from the mean Hb and Hc data of Til *et al.* (1972) also showed a significant decrease in the 6% group compared to the control group.

Hepatic thiamine concentrations determined in three rats from both the 6% dietary metabisulphite group and the corresponding diet control group were 8·8 ± 1·2 and 10·1 ± 0·7 μg/g wet weight (means ± SD), respectively. In spite of the addition of 50 ppm thiamine to the sulphited, but not to the control diet, SO_3^{--} mixed with the diet apparently caused a slight, although statistically non-significant ($P > 0·05$) reduction in mean hepatic thiamine concentration. It is assumed that the 3-wk treatment period provided sufficient time for the rat tissues to attain equilibrium with the thiamine intake rate since the turnover of thiamine in the rat has been characterized by a half-life of approximately 35 hr in the liver as well as the brain and heart (Sen & Cooper, 1976).

During the 3-wk feeding experiment, rats in the 6% metabisulphite group consumed considerably less feed than the controls and lost an average of 17 g body weight, while the control group gained an average of 13 g. (The rats ingesting 6% metabisulphite, but not the 1 and 2% groups, also exhibited traces of what appeared to be a dried, haem-like substance near their nostrils.) Our results are consistent with those of Til *et al.* (1972), who showed a decrease in food consumption and food efficiency in the 6% metabisulphite group. Spleen weight, expressed as a percentage of body weight, was statistically increased at the end of the feeding period in the 6% group; part of this increase was due to a decrease in body weight and part to an increase in the absolute weight of the organ. In spite of a decrease in food consumption in the 6% group, liver weight, expressed as a percentage of body weight, did not differ statistically from the controls. In the 1 and 2% metabisulphite groups, food consumption and weight gain over the treatment period were slightly decreased relative to the controls.

Tissue RS-SO_3^-

S-Sulphonate metabolites of tissues such as plasma, aorta and skin can be used as indicators of the relative level of systemic SO_3^{--} concentration; this has been demonstrated recently using the sulphite-oxidase-deficient rat model (Gunnison *et al.* 1981). In order to compare systemic SO_3^{--} exposures in various treatment groups of the sulphite-oxidase-deficient rat with those of normal rats ingesting SO_3^{--} mixed with the diet, tissue RS–SO_3^- concentrations were determined (Table 5). Also tabulated for each group is the

Table 4. *Biochemical and physiological parameters in rats after 21 days of treatment with 0–6% dietary sodium metabisulphite*

Dietary level of $Na_2S_2O_5$ (%)	Hb (g/100 ml blood)	Hc (%)	Hb/Hc	Prothrombin time (sec)	Hepatic thiamine (μg/g wet weight)
0	16·4 ± 0·6 (15)	49·3 ± 1·9 (15)	0·33 ± 0·01 (15)	14·5 ± 0·4 (5)	10·1 ± 0·7 (3)
1	16·0 ± 0·6 (8)	48·4 ± 1·1 (15)	0·33 ± 0·01 (8)	—	—
2	15·4 ± 0·9** (10)	45·9 ± 2·0*** (10)	0·34 ± 0·01 (10)	—	—
6	9·5 ± 2·6*** (12)	33·0 ± 6·3*** (12)	0·28 ± 0·03*** (12)	14·2 ± 0·3 (5)	8·8 ± 1·2 (3)

Hb = Haemoglobin Hc = Haematocrit

Values are means ± 1 SD for the numbers of rats indicated in brackets, and those marked with asterisks differ significantly (Student's *t* test) from the control group given diet without added $Na_2S_2O_5$: $^{**}P < 0.01$; $^{***}P < 0.001$.

approximate daily intake of exogenous SO_3^{--} and the classification of the hepatic level of sulphite-oxidase activity, i.e. normal or deficient.

In Table 5, aorta RS–SO_3^- concentrations represent the only complete set of tissue RS–SO_3^- data and show the most marked and consistent response to systemic SO_3^{--}. The endogenous concentrations observed in the diet control groups (pregnant and non-pregnant/normal sulphite oxidase) and the Mo control groups (pregnant/normal sulphite oxidase) are all approximately 1000 nmol/g dry weight. In the three sulphite-oxidase-deficient groups, elevation above this endogenous level increases progressively as exogenous SO_3^{--} intake increases from zero through 3·5 nmol/kg/day. The endogenous SO_3^{--} produced in NP/W rats is responsible for a considerable amount of aortic RS–SO_3^- production and is, in fact, approximately equivalent to the addition of 2% metabisulphite to the diet of rats with normal sulphite-oxidase activity. The pinna and plasma RS–SO_3^- concentration data, although incomplete, show the same trends as the aorta data. In the 1 and 2% metabisulphite groups, pinnas and aortas analysed after 35 days had mean RS–SO_3^- concentrations similar to those analysed after 21 days of treatment, indicating that tissue RS–SO_3^- concentration was in equilibrium with the rate of exogenous SO_3^{--} intake.

Comparison of tissue RS–SO_3^- concentrations in the NP/W/$SO_3^{--}{}_{(50)}$ and 6% dietary metabisulphite groups indicate that systemic SO_3^{--} concentrations were approximately the same in both groups in spite of the fact that exogenous SO_3^{--} intake was seven times lower in the former. This apparent inconsistency is readily explicable by the marked difference in sulphite-oxidase activity between the two groups. It is important to state here that we have shown recently in both *in vivo* and *in vitro* experiments that rat aorta possesses sufficient SO_3^{--}-reactive sites for the formation of approximately 20,000 nmol RS–SO_3^-/g dry weight. Thus, the SO_3^{--}-reactive sites in the aortas of the 6%-dietary metabisulphite and NP/W/$SO_3^{--}{}_{(50)}$ groups were not saturated when the rats were killed and presumably reflected the systemic SO_3^{--} concentration.

Although systemic SO_3^{--} exposure in the NP/W/$SO_3^{--}{}_{(50)}$ and 6% dietary metabisulphite groups was similar, the latter group was shown to be severely anaemic and to exhibit a possible slight depression of hepatic thiamine concentration as well, while the NP/W/$SO_3^{--}{}_{(50)}$ group was normal in these respects. It appears, therefore, that these effects correlate with the ingestion of diets containing high concentrations of SO_3^{--} and not necessarily with high levels of systemic SO_3^{--}.

Mammary adenocarcinoma

An unexpected finding during the course of this and a companion study was a low incidence of mammary adenocarcinoma in rats in the sulphite-oxidase-deficient (i.e. treatment) groups. These tumours were palpable externally and were discovered in rats that were 105–130 days old and had been on treatment for between 40 and 65 days; no tumours were detected in rats with normal sulphite-oxidase activity (PLC- and NP-diet controls and Mo controls). Table 6 shows a breakdown of the tumour incidence in various groups. Rats used in the dietary metabisulphite feeding study were on test for only 3 or 5 wk, exhibited no palpable mammary tumours, and were excluded from this tabulation. The data in Table 6 include non-pregnant animals that were part of a metabolism study (Gunnison *et al.* 1981) as well as pregnant and non-pregnant animals from the experiments reported in this paper. Since, during the course of these experiments, animals were killed over a range of treatment times for sulphite-oxidase assay, for determination of tissue RS–SO_3^- and/or for investigation of teratogenic effects, the criterion used to establish the number of animals at risk of developing mammary carcinoma was their residence in a specific control or treatment group for a minimum of 40 days; this was the earliest time at which a palpable tumour was detected. Of the rats thus determined to be at risk, at least 95% were killed before a maximum of 70 days residence time.

The tumour incidence shown in Table 6 shows a treatment-response but not a dose-response relationship with respect to systemic SO_3^{--}, although the numbers of animals at risk in each group are too small to draw a firm conclusion. Since no tumours were found in the diet control or Mo control groups, one might suspect that either systemic SO_3^{--}, sodium tungstate or systemic SO_3^{--} plus sodium tungstate is a necessary condition for tumorigenesis. Comparison of the tumour incidence in all treatment (sulphite-oxidase-deficient) groups combined (4/149) with the incidence in all control (normal sulphite oxidase) groups combined (0/143), using Fisher's exact test (Sokal & Rohlf, 1969), gives a probability of 0·066 (one-tailed test) that the observed distribution of tumours could have occurred by chance alone. If no assumptions are

Table 5. *Tissue* S-*sulphonate concentrations in experimental groups of sulphite-oxidase-deficient and normal rats*

Experimental group*	Experimental period (days)	Sulfite-oxidase activity	Mean exogenous SO_3^{--} intake (mmol/kg/day)	Mean $RS\text{–}SO_3^-$ concentration		
				Aorta (nmol/g dry weight)	Pinna (nmol/g wet weight)	Plasma (nmol/70 mg protein)
Pregnant females						
NP†	63	Normal	0	1083 ± 210 (6)	286 ± 49 (4)	21 ± 3 (3)
PLC†	63	Normal	0	1016 ± 514 (5)	—	13 ± 3 (3)
NP/W/Mo/SO_4^{--}(25)†	63	Normal	0	1268 ± 244 (6)	—	26 ± 8 (3)
NP/W/Mo/SO_4^{--}(50)†	63	Normal	0	948 ± 177 (4)	—	—
NP/W†	63	Deficient	0	6415 ± 3219 (12)	560 ± 158 (4)	63 ± 15 (9)
NP/W/SO_3^{--}(25)	63	Deficient	2·0	10,524 ± 2440 (7)	—	64 ± 10 (6)
NP/W/SO_3^{--}(50)	63	Deficient	3·5	16,532 ± 1401 (8)	1322 ± 200 (7)	91 ± 19 (6)
Non-pregnant females‡						
NP	21	Normal	0	958 ± 355 (4)	341 ± 76 (7)	—
Dietary SO_3^{--}: 1%	21 & 35	Normal	6	3100 ± 903 (6)	536 ± 128 (10)	48 (3)
2%	21 & 35	Normal	13	5953 ± 1219 (10)	691 ± 136 (10)	—
6%	21	Normal	25	13,729 ± 1557 (6)	1226 ± 285 (8)	110 ± 27 (6)

$RS\text{-}SO_3^-$ = *S*-Sulphonate

* See Table 1 for description of first seven experimental groups.
† Mean concentrations include previously reported data (Gunnison *et al.* 1981).
‡ Fed NP diet containing 0–6% $Na_2S_2O_5$.
Values for $RS\text{–}SO_3^-$ concentrations are means ± 1 SD for the numbers of rats indicated in brackets.

Table 6. *Incidence of mammary adenocarcinoma in experimental groups of sulphite-oxidase-deficient and normal rats*

Experimental group*	No. of rats with tumours/no. of rats at risk		
	Pregnant	Not pregnant	Total
PLC	0/28	0/29	0/57
NP	0/30	0/17	0/47
NP/W/Mo/SO_4^{--} (25) + (50)	0/31	0/8	0/39
	Sum for rats with normal sulphite oxidase...		0/143
NP/W	1/24	2/60	3/84
NP/W/SO_3^{--} (25)	1/19	0/2	1/21
NP/W/SO_3^{--} (50)	0/24	0/12	0/36
NP/W/SO_3^{--} (75)	0/7	0/1	0/8
	Sum for sulphite-oxidase-deficient rats...		4/149

* See Table 1 for description of experimental groups.

made *a priori*, the incidence of tumours in the treatment groups (4/149) should be compared with the incidence in only the NP-diet control (0/47). Similar statistical analysis indicates a probability of 0·33 that this distribution of tumours could have occurred by chance. Thus, statistically, the tumours observed in the treatment groups are not significant.

DISCUSSION

The sulphite-oxidase-deficient rat model used in these experiments is effective in producing relatively high levels of systemic SO_3^{--} from administration of small doses of exogenous SO_3^{--}. Even without exogenous SO_3^{--} administration there is evidence of generation of a substantial concentration of SO_3^{--} from endogenous sources. To duplicate these high levels of systemic SO_3^{--} in normal rats requires incorporation of large amounts of SO_3^{--} salts into the diet. It was demonstrated in this and previous studies that such treatment results in toxicity due to (1) decreased food consumption and efficiency, (2) reactions of SO_3^{--} with constituents of the diet and (3) irritation of the gut. Exposure of the human population to SO_3^{--} is more closely approximated by the conditions of low sulphite-oxidase level and low exogenous SO_3^{--} intake as observed in the sulphite-oxidase-deficient rat model, although admittedly this model is an exaggeration of the normal human situation. Nevertheless, the model does allow one to investigate the question of systemic SO_3^{--} toxicity without the complications produced by the administration of large doses of exogenous SO_3^{--}.

Since the administration of sodium tungstate is an essential element of this animal model, its toxicity is of major concern. A review of the literature reveals that the toxicity of tungstate has not been rigorously investigated. Schroeder & Mitchener (1975) administered 5 ppm sodium tungstate in the drinking-water of rats over their lifetime and observed a slight but statistically significant reduction in longevity; no other toxic signs were observed. Additional long-term testing with higher doses of sodium tungstate could not be found in the literature, although there are several reports of relatively short-term administration for the purpose of inhibiting molybdenum-containing enzymes. Other than sulphite oxidase, only two mammalian enzymes, xanthine oxidase and aldehyde oxidase, have been shown to contain functional Mo (Cohen, Fridovich & Rajagopalan, 1971). The latter two enzymes have similar substrate specificities, and one of these, aldehyde oxidase, is apparently absent in several species of mammals (Knox, 1946) and is present in small amounts in the rat (Johnson *et al.* 1974). Congenital deficiency of xanthine oxidase has been described in humans and does not cause serious health impairment (Watts, Engelman, Klinenberg, Seegmiller & Sjoerdsma, 1964). Thus, overt toxicity due to decreased activity of xanthine oxidase or aldehyde oxidase was not anticipated in this sulphite-oxidase-deficient rat model. Further, with the exception of the progeny of rats maintained on 400 ppm W (Johnson & Rajagopalan, 1976), Rajagopalan and coworkers have not reported any obvious deleterious effects on the health of rats with W-induced Mo deficiency. In our studies, aside from the higher incidence of largely transient diarrhoea which was attributable to a lack of fibre in the diet, sulphite-oxidase-deficient animals appeared outwardly as healthy as control animals.

The results of our experiments clearly demonstrated that anaemia can be produced by high concentrations of SO_3^{--} in the diet and/or the gut, and is not attributable to the action of systemic SO_3^{--}. Rats in the NP/W/SO_3^{--} (50) group, which exhibited evidence of systemic SO_3^{--} levels similar to those of rats ingesting 6% dietary metabisulphite, were essentially identical in Hc and in Hb concentration to their matched control groups, while the 6%-dietary metabisulphite rats were severely anaemic. Although the direct cause of this anaemia was not investigated, it seems probable from information recently provided by Terao, Marui, Tanaka & Nakao (1980) that the degradation of cyanocobalamin in the diet and/or gut was responsible. These authors demonstrated that the half-life of cyanocobalamin in an aqueous solution of bisulphite at pH 5·3 and 25°C was 38 min when there was a 100:1 molar ratio of bisulphite to cyanocobalamin.

It has been adequately demonstrated by other investigators that SO_3^{--}, when premixed with the diet and stored, is capable of significant destruction of the dietary thiamine content. This destruction is a function of SO_3^{--} concentration, and of time and temperature of storage (Fitzhugh *et al.* 1946; Til *et al.* 1972). Thiamine can also be destroyed in the gut by high concentrations of SO_3^{--} (Lhuissier, 1966). It is not clear from these studies, however, whether or not

significant amounts of thiamine can also be destroyed systemically by SO_3^{--}. Our experiments with sulphite-oxidase-deficient rats show that, under conditions of high systemic SO_3^{--} concentrations produced without the incorporation of SO_3^{--} into the diet (NP/W/$SO_3^{--}$$_{(50)}$ group), hepatic thiamine concentration is not decreased. To produce equally elevated systemic SO_3^{--} concentrations in normal rats requires the addition to the diet of 6% metabisulphite or the equivalent; this mixture is debilitating to adult rats. Dietary SO_3^{--} concentrations considerably lower than this, particularly when not stored at low temperatures prior to use, are capable of producing toxic signs which can be eliminated by concurrent thiamine administration (Bhagat & Lockett, 1964; Fitzhugh *et al.* 1946). We, conclude therefore, that there is no evidence for the systemic destruction of thiamine in rats, even under conditions of extreme systemic SO_3^{--} exposure.

There were no dose-related trends in one-stage prothrombin times among the groups tested, indicating that neither elevated systemic SO_3^{--} concentrations nor high concentrations of SO_3^{--} mixed with the diet affected vitamin K function. Inhibition of function had been suspected from preliminary experiments in which grossly sulphite-oxidase-deficient rats had exhibited dried, haem-like secretions on their snouts. Sulphite does react readily, although reversibly, with vitamin K_3 (menadione), a synthetic vitamin K supplement in the NP diet. At physiological pH, the pK of the reaction favours the bisulphite adduct over the free form, and at a molar ratio of approximately 1, essentially all of the menadione is in the bisulphite form (Vire, Patriarche & Christian, 1979). Further, menadione must be in the dissociated state for epoxidation to occur, a reaction which is apparently required for prothrombin formation (Sadowski, Schnoes & Suttie, 1977). It seems reasonable that in the tissues of the sulphite-oxidase-deficient rats used in our experiments, menadione would exist largely as the bisulphite adduct and would, therefore, be inactive. However, since coprophagy was not prevented in any of the rats in our experiment, vitamin K deficiency would not be expected to occur unless SO_3^{--} also inhibited its synthesis by micro-organisms in the intestine or its subsequent absorption after coprophagy. Thus, a combination of inhibition of intestinal vitamin K synthesis or absorption, and inactivation of dietary menadione would be required to produce vitamin K deficiency. The data do not support such a hypothesis. It seems more likely that the dried, haem-like secretions observed on the snouts of some SO_3^{--}-treated or sulphite-oxidase-deficient rats, in previous as well as the current experiments, accumulated because of an absence in these rats of their normal grooming behaviour.

The rationale for our investigation of the effect of systemic SO_3^{--} on RBC-GSH concentration is based on the reaction of SO_3^{--} with oxidized glutathione (GSSG), forming one molecule of GSH and one molecule of glutathione *S*-sulphonate ($GSSO_3^-$). This reaction occurs readily *in vitro* (Gunnison & Benton, 1971) and almost certainly occurred to some extent in the sulphite-oxidase-deficient rats used in these experiments, although neither body tissues nor urine were analysed for this specific metabolite. Oxidized glutathione concentration would presumably be decreased by its sulphitolysis which, assuming an equilibrium between GSSG and GSH, would ultimately result in the decreased concentration of GSH. Reduced glutathione was measured rather than GSSG because of the analytical difficulty of measuring the latter. Our results show that there was no SO_3^{--}-induced reduction of RBC-GSH. In general, the ability of rats to maintain normal levels of GSH in spite of great perturbing influences suggested to us at the outset that a significant change in RBC-GSH concentration would be observed only if the sulphitolysis of GSSG and subsequent regeneration from GSH were rapid reactions of substantial magnitude.

The most provocative finding of this study is that of mammary adenocarcinoma in young, sulphite-oxidase-deficient females. Since the number of NP-diet control animals at risk was relatively low, and the incidence of tumours in the treatment groups was also low (approximately 2·5%), the probability of this distribution of mammary carcinoma occurring by chance alone is too large to justify a conclusion of statistical significance. However, these mammary carcinomas occurred in rats at an age (less than 5 months) when their spontaneous development would be a very rare event. Thus, it is highly likely that the observed mammary adenocarcinomas are, in fact, treatment related. Although there are no historical data available on the effect of the NP diet on tumour development, this diet has been available commercially in its present formulation for at least 13 years without reported effects on increased incidences of carcinoma in young adult female rats (personal communication, ICN Nutritional Biochemicals, 1980).

If, in fact, the mammary adenocarcinomas are treatment related, their aetiology is of interest. Since systemic SO_3^{--} is present at levels significantly above normal endogenous levels in all the treatment groups, as evidenced by tissue RS–SO_3^{--} data, SO_3^{--} may play a role in the observed tumorigenesis. The possibility that SO_3^{--} alone could have produced these tumours seems remote, but nevertheless, cannot be ruled out. Til *et al.* (1972), in their investigation of the chronic toxicity of SO_3^{--}, fed metabisulphite at a maximum concentration of 2% in the diet to three generations of rats and reported no treatment-related tumours of any type. It is apparent from our experiments that this 2% feeding regimen is approximately equivalent to the NP/W treatment in terms of systemic SO_3^{--} concentration. Although the total number of female rats in the 2% dose level in the experiments of Til *et al.* (1972) is not clear, it is unlikely that the numbers are large enough to rule out, at an acceptable level of significance, the possibility of a causal role for SO_3^{--} in the production of this low incidence of mammary carcinoma. There have been many other studies of chronic SO_3^{--} toxicity in rats without evidence of SO_3^{--}-induced tumours (Cluzan *et al.* 1965; Fitzhugh *et al.* 1946; Lanteaume *et al.* 1965; Lockett & Natoff, 1960). In none of these, however, was the rate of SO_3^{--} intake great enough to be equivalent to the systemic SO_3^{--} concentration observed in the NP/W group.

Previous investigation of the carcinogenicity of sodium tungstate and other tungsten compounds has been limited. The chronic feeding study of Schroeder

& Mitchener (1975) was mentioned previously. Similar studies at higher doses were not found in the literature. Related studies with sodium tungstate and other tungsten compounds have been designed either to investigate distribution and elimination (Kinard & Aull, 1945) or toxicity following tracheal instillation (Delahant, 1955; Schepers, 1955a,b,c). These studies used small numbers of animals and no tumours were reported.

The data generated in our experiments are insufficient in themselves to demonstrate conclusively that the sulphite-oxidase-deficient rat is at greater than normal risk of developing breast cancer. It is, therefore, premature to speculate on the possible roles of the various elements of the sulphite-oxidase-deficient rat model (i.e. high W, Mo deficiency, systemic $SO_3^{=}$, NP diet) in the production of mammary adenocarcinoma in the Wistar rat. Indeed, before attempting to dissect from the model the elements critical to the pathogenesis of these mammary tumours, we feel that the reproducibility of tumorigenicity in the model should be investigated.

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SHORT PAPER

ANALYSE DE LA NITROSODIETHANOLAMINE DANS LES PRODUITS DE L'INDUSTRIE COSMETIQUE

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Résumé—Les auteurs décrivent une technique d'analyse de la *N*-nitrosodiéthanolamine (NDELA) dans les produits cosmétiques. Après évaporation de l'eau en présence d'un inhibiteur de la réaction de nitrosation l'essai est purifié sur une colonne de silice. La détermination est réalisée par chromatographie liquide haute performance couplée au détecteur TEA. La méthode est appliquée à la recherche de NDELA dans quelques échantillons de matières premières et de produits finis de l'industrie cosmétique.

Abstract—The method described for determining *N*-nitrosodiethanolamine (NDELA) in cosmetic products involves the evaporation of water from the sample in the presence of a nitrosation-inhibiting agent, followed by purification of the sample on a silica-gel column. NDELA levels are then determined by high-pressure liquid chromatography coupled to a TEA detector. This procedure has been used to estimate NDELA in several raw materials and finished products from the cosmetics industry.

Introduction

La présence de composés *N*-nitrosés dans notre environnement préoccupe depuis une vingtaine d'années les hygiénistes. Ces composés dont certains ont une action carcinogénique marquée qui n'est plus à démontrer chez l'animal (Magee et Barnes, 1967) ont été effectivement recensés dans les aliments et les boissons, l'air et l'eau, certains produits manufacturés tels que des herbicides en solution aqueuse, les huiles de coupe synthétiques et les cosmétiques (Fine, 1978). La cancérogénicité de la nitrosodiéthanolamine a été établie par Druckrey, Preussmann, Ivankovic et Schmähl (1967) puis confirmée récemment par Hilfrich, Schmeltz et Hoffmann (1978). Il est donc utile d'évaluer son taux dans les produits cosmétiques.

Jusqu'à ces cinq dernières années, des difficultés analytiques ont ralenti la recherche des nitrosamines non volatiles alors que celle des composés volatils connaissait un développement important. La récente extension de la chromatographie liquide à haute performance et le couplage de celle-ci au détecteur TEA permet la détermination de ces composés.

Ce détecteur, dont le principe est basé sur la chimiluminescence des oxydes d'azote, est sensible et hautement spécifique. Il réalise en effet des analyses rapides et sûres moyennant quelques précautions concernant la préparation de l'échantillon.

Nous rapportons ici une technique utilisant cet appareillage pour évaluer la nitrosodiéthanolamine dans les échantillons préalablement purifiés.

Méthodes expérimentales

Réactifs. La nitrosodiéthanolamine et la nitrosodiisopropanolamine sont synthétisées au laboratoire selon la méthode de Jones et Wilson (1949). Leur pureté est ensuite contrôlée par chromatographie liquide couplée au détecteur TEA dans les conditions décrites ci-dessous et à l'aide d'un standard de nitrosodiéthanolamine obtenu auprès du service analytique de Thermo Electron Corp. (Waltham, MA, É-U).

Les solvants organiques et les produits chimiques entrant dans la technique sont de qualité analytique et ont été obtenus auprès de E. Merck AG (Darmstadt RFA). Les solvants sont redistillés en montage de verre. La silice 60 (E. Merck AG, réf. 7734) est activée préalablement à l'emploi durant 24 heures à 100°C.

Appareillage. Les déterminations analytiques sont réalisée à l'aide d'un système chromatographique en phase liquide composé d'une pompe Model 110 A (Altex Corp., Berkeley, CA, É-U), d'un injecteur Model 7120 (Rheodyne, Berkeley, CA) et d'une colonne μ porasil (300 × 3,9 mm) (Waters Associates, Milford, MA, É-U). La phase mobile est un mélange acétone–isooctane (35:65, v/v) au débit de 1,5 ml/min. Le chromatographe est couplé à un détecteur TEA 502/LC (Thermo Electron Corp.) équipé d'un piège double maintenu à −72°C (carboglace–éthanol). Un troisième piège est porté à −161°C (mélange isopentane–azote liquide). La température du four de pyrrolyse est maintenue à 550°C. La purification des échan-

tillons est réalisée dans des colonnes de verre (dimensions internes 15 × 300 mm).

Méthodologie. L'échantillon homogénéise (10 g) sont placé dans un ballon. L'acide ascorbique (0,250 g) est ensuite ajouté comme inhibiteur de formation de la nitrosodiéthanolamine durant le traitement, et 360 ng de nitrosodiisopropanolamine sont ajoutés à l'essai en tant que standard interne. L'eau est évaporée sous vide à 50°C. L'échantillon sec est ensuite placé une nuit au dessiccateur sous vide phosphorique. Le résidu pâteux est repris dans l'acétate d'éthyle, filtré, concentré à 5 ml avant d'être déposé sur la colonne de purification. Celle-ci est composée de 15 g de gel de silice 60 préalablement suspendu dans l'acétate d'éthyle. Le sulfate de sodium anhydre (1 g) est déposé à la partie supérieure de la phase puis est recouvert de solvant.

La colonne est éluée successivement par 40 ml d'acétate d'éthyle, 20 ml d'un mélange à 5% puis enfin de 20 ml d'un mélange à 15% d'acétone dans l'acétate d'ethyle. Les 20 premiers millilitres sont rejetés. L'éluat (60 ml) est concentré sous vide à 50°C jusqu'à un volume de 5 ml puis sous courant d'azote jusqu'à 0,5 ml. Pour l'analyse 20–100 µl sont injectés dans le chromatographe.

Resultats et Discussion

Les chromatogrammes obtenus dans les conditions expérimentales décrites ci-dessus sont facilement interprétables et quantifiables (Fig. 1). Le rendement de l'analyse calculé par rapport à la nitrosodiisopropanolamine ajoutée est voisin de 60%. La limite de détection de 10 ppb est atteinte aisément.

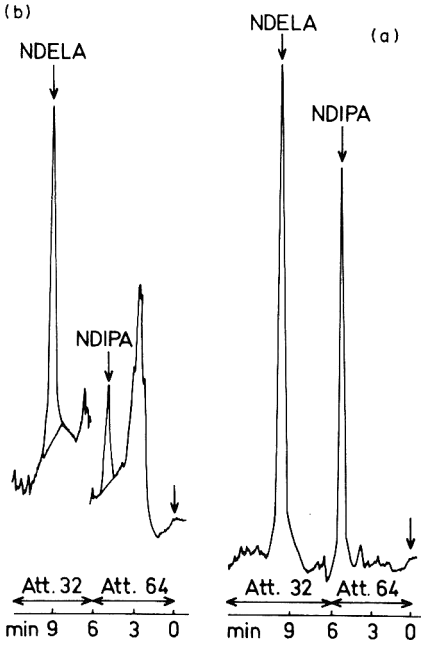

Fig. 1. Chromatogrammes obtenus par HPLC–TEA (a) après injection de 50 µl d'un mélange de standard de la nitrosodiéthanolamine (NDELA; 32,5 ng) et de la nitrosodiisopropanolamine (NDIPA; 45 ng) dans l'acétate d'éthyle, et (b) après injection d'un concentrat obtenu à partir du shampooing.

Tableau 1. *Evaluation du taux de la N-nitrosodiéthanolamine (NDELA) dans quelques matières premières et produits finis cosmétiques*

Enchantillon	NDELA (ng/g)*
Matières premières	
Laurylsulfate de triéthanolamine	12,8
Alkylaminoethersulfate (polyoxyethylene) de triéthanolamine	ND
Diéthanolamide d'acide gras de coprah	ND
Produits finis	
Shampooings ou bases de shampooings (anioniques)	
155/17-1	ND
155/17-2	ND
155/17-3	ND
155/17-4	308
155/17-5	46
155/17-6	ND
155/17-9†	4113
Bain moussant (type anionique)	
155/17-7	26,5
Savon liquide (type anionique)	
155/17-11	<20
Crème (émulsion huile/eau)	
155/17-10	ND

ND = Non détecté

*Limite de détection, 10 ng/g.

†Base de shampooing formulée avec du laurylsulfate de triéthanolamine, de la diéthanolamide d'acide gras de coprah et un conservateur susceptible de générer des ions nitrites (5-bromo-5-nitro-1,3-dioxane).

Dans un précédent travail, Fellion, de Smedt et Brudney (1979) ont montré l'efficacité de l'acide ascorbique en tant qu'inhibiteur de la réaction de nitrosation dans les milieux cosmétiques. Nous nous sommes placés dans des conditions analogues afin de nous garantir contre toute formation de nitrosodiéthanolamine durant l'analyse. Fan, Goff, Song, Fine, Arsenault et Biemann (1977) ainsi que de nombreux autres auteurs utilisent dans ce but le sulfamate d'ammonium. Ce composé cependant n'est un bon inhibiteur qu'en milieu acide, ce qui ne correspond pas au cas précis des cosmétiques pour lesquels le pH avoisine la neutralité.

Nous avons tenté d'optimiser les conditions d'emploi du détecteur TEA. La température du four de pyrrolyse maintenue à 550°C est insuffisante pour briser la liaison C–NO des composés *C*-nitrosés éventuellement présents. Nous éliminons ainsi un certain nombre de possibilités de fausses réponses positives du détecteur. L'emploi de trois pièges en série aux températures de −72°C, −72°C et −161°C permet de retenir efficacement les produits de pyrrolyse. Nous avons constaté une diminution du bruit de fond de l'appareil ainsi qu'une augmentation de la sensibilité par rapport à un système de double pièges à −72°C.

Nous avons utilisé cette technique pour la recherche de nitrosodiéthanolamine dans des matières premières et des produits finis de l'industrie cosmétique. Les résultats rassemblés dans le Tableau 1 sont corrigés par rapport au rendement. Ils montrent une pollution faible voire non détectable dans des pro-

duits cosmétiques formulés avec un rationnel qui élimine l'apport exogène d'entités nitrosantes minérales (nitrates ou nitrites) ou organiques (conservateurs du type 2-bromo-2-nitropropan-1,3-diol ou 5-bromo-5-nitro-1,3-dioxane).

Il apparait clairement que l'utilisation de techniques analytiques viables comme celle qui est décrite ainsi que la prise en considération des causes possibles de contamination des produits cosmétiques par la nitrosodiéthanolamine devraient permettre de mieux maitriser cet éventuel problème.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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CAPRYLIC ACID

Synonyms: Octanoic acid; *n*-octoic acid; C-8 acid; 1-heptanecarboxylic acid; octylic acid.
Structure: $CH_3 \cdot [CH_2]_6 \cdot COOH$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to occur frequently in the essential oils of *Cupressus torulosa*, *Cryptomeria japonica*, *Andropogon iwarancusa*, *Cymbopogon javanensis*, camphor, nutmeg, lemongrass, lime, tobacco (flowers), Artemisia herba-alba, camomile, hops and other plants, and to be found in apple aroma, coconut oil (as glyceride) and wine (as an ester), and (both free and esterified) among the constituents of petitgrain lemon oil (*Fenaroli's Handbook of Flavor Ingredients*, 1975). Also found in apple, banana, cherries, citrus fruits, grape, papaya, peach, pear, raspberry, blackberry, strawberry, sauerkraut, tomato, cinnamon, mint, breads, milk products including cheeses, heated lamb, beef and pork, beer, grape brandy, rum, whisky, cider, sherry, wine, cocoa, coffee, tea, roasted peanuts and pecans, potato chips (American), soya beans, arctic bramble, cloudberry, passion fruit, mushroom, wild marjoram, plum brandy and pear brandy (CIVO–TNO, 1977).
Preparation: By oxidation of octanol (*Merck Index*, 1976).
Uses: In public use since the 1950s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·001	0·0001	0·0001	0·002
Maximum	0·015	0·0015	0·005	0·1

Analytical data: Infra-red curve, RIFM no. 77–62.

Status

Caprylic acid was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 182.3025) and was included by the Council of Europe (1974) at a level of 100 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on caprylic acid, and the toxic effects of the acid have recently been summarized (National Institute for Occupational Safety and Health, 1977). The Federation of American Societies for Experimental Biology (1974) has stated that "There is no evidence in the available information on caprylic acid that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that are now current or that might reasonably be expected in future". This evaluation was based in part on a scientific literature review covering 1920–1972 (Franklin Institute Research Laboratories, 1972). The GRAS status of caprylic acid has been affirmed (Food and Drug Administration, 1977 & 1978). CAS Registry No. 124–07–2.

Biological data†

Acute toxicity. The acute oral LD_{50} in rats was reported as 10·08 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. Caprylic acid applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately to severely irritating (Moreno, 1977). Tested at 1% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977). Caprylic acid was among the most irritating in a series of fatty acids, ranging in chain length from C_3 to C_{18}, when applied daily *in vivo* under conditions of occlusive patch testing to the skin of human males; the acid

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology*, 1981, **19**, no. 1 (pp. 97–116).
†Literature searched from 1972 through June 1978.

had been stored in propanol and was administered in propanol solution (Stillman, Maibach & Shalita, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77-62) was tested at a concentration of 1% in petrolatum and produced no sensitization reactions (Kligman, 1977).

Transport and absorption. It has been demonstrated that caprylic acid, in Ringer's solution containing other lipids and bile salt, can be absorbed directly from the intestine of the rat (Carlier & Bezard, 1975). The absorption may involve active transport (Bloch, Dennhardt, Lingelbach & Lorenz-Meyer, 1972; Bloch, Haberich & Lorenz-Meyer, 1972). The rat blood-brain barrier was permeable to caprylic acid (Oldendorf, 1972 & 1973). Serum binding of caprylic acid was unchanged from control values in chickens with oestradiol 3-benzoate-induced lipaemia (Rudman, Hollins, Bixler & Mosteller, 1972). Lung tissue did not exhibit a high affinity for caprylic acid, as determined by the administration of the ^{11}C-labelled compound to mice (Fowler, Gallagher, MacGregor & Wolf, 1976). *In situ* vaginal absorption of [^{14}C]caprylic acid has been demonstrated in rabbits (Hwang, Owada, Suhardja, Ho, Flynn & Higuchi, 1977).

Metabolism. Caprylic acid is a naturally occurring fatty acid which may be biosynthesized *de novo* from malonyl CoA and acetyl CoA (Cunningham, 1978); catabolism of this fatty acid involves β-oxidation to acetyl CoA. Among the many changes in blood chemistry that may occur in hepatic coma and encephalopathy or in cirrhosis of the liver, an increase in serum caprylic acid levels has been reported (Brunner & Siehoff, 1976; Rabinowitz, Staeffen, Blanquet, Vincent, Terme, Series & Myerson, 1978).

Biochemical interactions. In the liver, caprylic acid increased gluconeogenesis in both control and thiamine-deficient rats and reduced concentrations of amino acids, except glycine, in control and diabetic rats (Paquet, Mackerer & Mehlman, 1972). The depletion of liver ATP was demonstrated following rapid iv administration of caprylic acid to rats (Bässler, Hassinger & Ackermann, 1977). Postprandial biosynthesis of cholesterol was not observed in rats when [^{14}C]caprylic acid was used as a precursor (Fears & Morgan, 1976).

Caprylic acid was one of several fatty acids that, together with bile acids, were studied in human volunteers for their effect on pancreatic and gall-bladder function (Malagelada, DiMagno, Summerskill & Go, 1976). Its perfusion in dogs with a chronic gastric or pancreatic fistula was not accompanied by increased pancreatic output (Meyer & Jones, 1974). Depending on the age of the neonatal rat, caprylic acid either inhibited or had no effect on the release of glucagon from the pancreas (Edwards, Asplund & Lundqvist, 1972). It was found to be a weak stimulant of pancreatic-enzyme secretion in another study in rats (Nakabou & Hagihira, 1972).

Caprylic acid altered the biological effects of [^{14}C]deoxycholic acid in rats, with respect to absorption of the bile acid, water and electrolytes (Lamabadusuriya, Guiraldes & Harries, 1975); also in the rat, it has been shown to affect the absorption and distribution of vitamin K_1 in lymphatic fluid and bile (Hollander & Rim, 1978), and to inhibit the intestinal absorption of L-tryptophan (Inui, Yamakita & Hori, 1976). Its transport especially to the liver and brain, and its absorption can be enhanced in mice by administration of pyrithioxine (Endo & Uchiyama, 1972).

Nutrition. Growth improvement and control of fattening were reported after caprylic acid, either alone or mixed with other fatty acids, had been fed for 25 days to immature rats receiving various types of diet (Aurousseau, De Groot, Duboisset & Bejot, 1972; Aurousseau, Duboisset, Bejot & Pacrot, 1972).

Pharmacology. Flavour thresholds were determined for caprylic acid in man, using solutions of acids buffered to three different pH values (Baldwin, Cloninger & Lindsay, 1973).

Mutagenesis assay. Caprylic acid, tested at 98%, did not exhibit genetic activity in a series of *in vitro* microbial assays with and without metabolic activation (Litton Bionetics, Inc., 1976). The microorganisms used were *Salmonella typhimurium* and *Saccharomyces cerevisiae.*

Anti-tumour activity. Caprylic acid showed no antitumour activity against Ehrlich ascites carcinoma in female ddY mice when given ip in a normal saline–Tween 80 suspension at 400 mg/kg/day for 5 days (Nishikawa, Okabe, Yoshimoto, Kurono & Fukuoka, 1976). Treatment was initiated 24 hr after tumour implantation with 7-day-old Ehrlich ascites cells ip. The treated animals showed a slight gain in body weight compared to the controls but two of the six treated animals died, while there were no deaths in the control group.

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ETHYL HEPTOATE

Synonyms: Ethyl heptanoate; ethyl heptylate; heptanoic acid, ethyl ester; ethyl oenanthate.
Structure: $CH_3 \cdot [CH_2]_5 \cdot COO \cdot CH_2 \cdot CH_3$.
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Reported to be found in apples, citrus fruits, grape, pineapple, strawberry, milk and milk products, hop oil, beer, grape brandy, rum, whisky, passion-fruit wine, sherry, wine, cocoa, tea, roasted filberts, olives, passion fruit and bantu beer (CIVO–TNO, 1977).
Preparation: By direct esterification of ethanol with *n*-heptanoic acid using azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·003	0·04
Maximum	0·05	0·01	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 76–101; infra-red curve, RIFM no. 76–101.

Status

Ethyl heptoate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was listed by the Council of Europe (1974) with an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on ethyl heptoate, and the Joint FAO/WHO Food Standards Programme (1973) has given the ester a conditional ADI of 0–1 mg/kg. CAS Registry No. 106–30–9.

Biological data*

Acute toxicity. The acute oral LD_{50} for rats was found to be > 34·64 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in rabbits was reported as > 5 g/kg (Moreno, 1977).

Subacute toxicity. When ethyl heptoate was fed in the diet to male and female rats for 13 wk, no macroscopic or microscopic effect was noted at 10,000 ppm, the highest level tested (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Irritation. Ethyl heptoate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–101) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1976).

Teratology studies. Ethyl heptoate (0·3 μl/larva) was found to be both toxic and melanogenic to larvae of the housefly, *Musca domestica* (Quraishi, 1972).

Pharmacology. Ethyl heptoate was incubated (at 2·78%) in a synthetic formulation developed as a replacement for a fermented egg product that attracts coyotes and repels deer (Bullard, Shumake, Campbell & Turkowski, 1978).

Nutrition. When ethyl heptoate was fed to chicks at 5% in the diet, medium energy availability (51%) was reported (Yoshida, Morimoto & Oda, 1970).

Metabolism. Esters of higher-molecular-weight fatty acids, such as ethyl heptoate, are thought to be readily hydrolysed to the corresponding alcohols and acids, which are then further metabolized (Fassett, 1963).

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ETHYLHEXYL PALMITATE

Synonyms: 2-Ethylhexyl palmitate; octyl palmitate; palmitic acid, 2-ethylhexyl ester; hexadecanoic acid, 2-ethylhexyl ester.

Structure: $CH_3 \cdot [CH_2]_{14} \cdot COO \cdot CH_2 \cdot CH(CH_2 \cdot CH_3) \cdot [CH_2]_3 \cdot CH_3$.

Description and physical properties: Clear, almost colourless liquid.

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

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·003	0·04
Maximum	0·05	0·01	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 77–478; infra-red curve, RIFM no. 77–478.

Status

Ethylhexyl palmitate is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 29806–73–3.

Biological data*

Acute toxicity. But the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1978).

Irritation. Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, ethylhexyl palmitate was slightly to moderately irritating (Moreno, 1978). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–478) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

Pharmacology. At a 10% concentration, ethylhexyl palmitate greatly increased the porosity of petroleum jelly to oxygen and carbon dioxide, as shown in human skin respiration tests (Jacobi, 1970).

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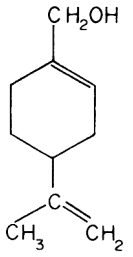
*Literature searched from 1962 through 1979.

PERILLA ALCOHOL

Synonyms: *p*-Mentha-1,8-dien-7-ol; 4-isopropenyl-cyclohex-1-ene-1-methanol; cyclohex-1-ene-1-methanol, 4-(1-methylethenyl); dihydrocuminyl alcohol; perillyl alcohol.
Structure:



Description and physical properties: Colourless to pale-yellow liquid.
Occurrence: The *d*- and *l*-forms occur naturally in the essential oils of ginger grass, *Juniperus sabina* L., East Indian geranium and others; the *l*-form is found in lavandin and bergamot, and the *d*-form in caraway (*Fenaroli's Handbook of Flavor Ingredients*, 1975). Perilla alcohol is also reported to occur in cranberries, bilberries and mint and hop oil (CIVO–TNO, 1977).
Preparation: By the hydrogenation of perilla aldehyde, followed by distillation.
Uses: In public use since the 1960s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·003	0·0005	0·0015	0·05
Maximum	0·05	0·005	0·015	0·4

Analytical data: Gas chromatogram, RIFM no. 77–122; infra-red curve, RIFM no. 77–122.

Status

Perilla alcohol was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. CAS Registry No. 536–59–4.

Biological data*

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·1 g/kg (1·7–2·6 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. Perilla alcohol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was severely irritating (Moreno, 1977). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–122) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

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Review Section

DOSE-RESPONSE FUNCTIONS IN CARCINOGENESIS AND THE WEIBULL MODEL

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Abstract—The Weibull model for dose-response functions in carcinogenesis is explored using published results from many large experiments with animals. Three conclusions emerge: the Weibull model fits these data in the statistical sense; the one-hit model is not an appropriate model; experimental results appear to fall into categories which can be labelled numerically according to the value of the shape parameter in the Weibull model.

Results from experiments with laboratory animals exposed (*via* ingestion or inhalation) to carcinogens over their entire lifetimes are now available in sufficient quantities to encourage the hope of finding some order among these results—perhaps even finding categories into which they fall. The outcome of each of these experiments is described by its dose-response pattern which relates the observed tumour rates (proportions) to the administered doses of the carcinogen. Some of these experiments have produced a dose-response pattern which indicates little or no effect of the carcinogen on the tumour rate at low doses, followed by a sudden rise in the tumour rate at higher doses. Other experiments have produced a pattern which indicates a sharp rise in the tumour rate at low doses of the carcinogen followed by a partial levelling off of the tumour rate at higher doses. Yet other experiments have produced intermediate patterns. The problem of finding an order among these patterns is further complicated by the inherent statistical variation in the observed tumour rates, especially when the numbers of animals at risk are not large.

The motivation for this study of the literature and the outline for this article is as follows. The problem of ordering the experiments would be simplified if there were some simple mathematical model which statistically fits the observed dose-response pattern from each experiment. If so, the estimated parameters in that model would contain all of the essential information in that experiment. Then one might hope to find an order among all the carcinogenesis experiments by comparing the values of their estimated parameters. Ideally, this order would be related to the severity of the carcinogens at the low doses of human interest.

The mathematical model to be used here is the Weibull model. (Johnson & Leone, 1964; the statistical details may be found in the Appendix.) There are several good features to the Weibull model and one poor feature. The good features are listed below.

(i) It fits the observed data in the statistical sense for a wide variety of studies.
(ii) It is simple to apply, requiring only weighted least squares on a desk calculator rather than a special program on a computer.
(iii) Although the Weibull model assumes that there is no threshold in the dose-response function, it does fit data with an apparent threshold.
(iv) At the other extreme, it also allows for concave dose-response functions.
(v) The simple form of the Weibull model is easily generalized to include both the dose of the carcinogen and the time to the kill (or the time to a tumour).
(vi) The important finding of Druckrey (1967) concerning the relation between the dose and the time to a tumour is implied by the Weibull model.
(vii) It is easily generalized to include a simultaneous analysis of several studies which share a common value of the critical parameter.

The poor feature of the Weibull model is that the critical parameter for ordering the dose-response functions has no apparent physiological interpretation at present.

The Weibull model has three parameters: α, β and m. The background tumour rate determines the value of α. The parameter β is primarily a scale parameter; that is, it depends on the units for the dose (ppm, for example). The parameter m depends on the shape of the dose-response function, and it is the critical one for this study.

To explore further the parameter m, suppose the true tumour rate at some high dose (like the maximum tolerated dose) is 0·63 and that the true background tumour rate is zero. Figure 1 shows the shapes of the dose-response functions for various values of the parameter m. Clearly, small values of m (like 0·5) correspond to dose-response functions with high tumour rates even at very low doses. Very high values of m (like 6·5) correspond to dose-response functions with very low tumour rates at very low doses, the practical equivalent of a threshold.

One useful concept in low-risk assessment is that of the virtual safe dose. This is usually taken as the life-

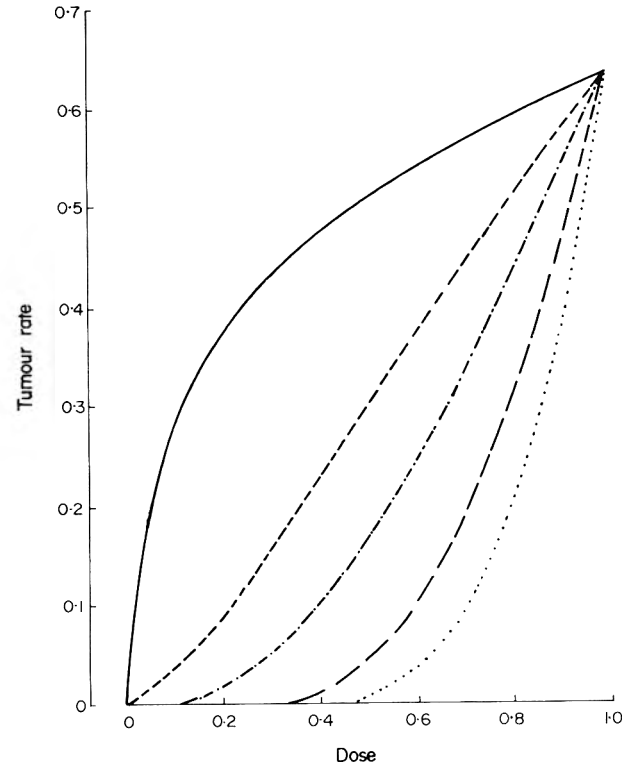

Fig. 1. Graphs of the Weibull model for various values of the parameter m: 0·5 (——), 1·5 (- - - - -), 2·5 (— · — · —), 4·5 (— — —), 6·5 (· · · · · ·).

time dose corresponding to a one-in-a-million lifetime risk of a cancer. If, for example, everyone in the USA ingested a substance carcinogenic for man at this dose for his entire life, then there would be three cancers per year caused by this carcinogen in the USA.

Table 1 gives the virtually safe doses (the VSD's) for various values of m for the situation in Fig. 1. For example, when m = 0·5, the VSD is about ten orders of magnitude lower than the dose which produces a tumour rate of 0·63 ($TD_{0·63}$). Notice the sharp increase in the VSD as m increases from 0·5 to 1·5 and higher. The relation given in Table 1 changes only very slightly for other values of the true background tumour rate and other values of the true tumour rate at the highest dose. Thus, it is essentially only the value of m which determines the risk at very low doses under the Weibull model.

The initial rule for including the results from an experiment for consideration and analysis in this article is as follows. There should be at least three positive doses plus a control group. At least two of these positive doses should produce observed tumour rates which are greater than the observed tumour rate for the control group. With less than three positive doses, there is no way to check the fit of the Weibull model to the data. With only one response clearly greater than that of the control group, the statistical error in the estimated value of the critical parameter m is extremely large. At the time of writing, all the studies meeting this rule and known to the author are included here. Undoubtedly, there are others.

There is no study with saccharin meeting this rule, because none has produced two responses clearly greater than the control. We must wait until the end of 1981 for the completion of the current large experiment with about 2000 Sprague–Dawley male rats.

The next problem is to define the pathological endpoint. There are two popular options: a malignant tumour or any tumour (benign or malignant). The rule here is to take a malignant tumour as the endpoint, but there is one important exception. Many of the best studies (in the sense of defining a dose-response function) deal with the liver of the mouse. Pathologists appear to vary greatly in their determination of a malignancy in the liver of a mouse (as revealed in the EPA cancellation hearings on heptachlor and chlordane (Environmental Protection

Table 1. *The relationship between the virtually safe dose (VSD; representing a risk of $1/10^6$) and the value of the parameter m in the Weibull model*

Weibull model parameter m	Virtually safe dose
0·5	$3·2 \times 10^{-10} \times TD_{0·63}$
1·5	$1·0 \times 10^{-4} \times TD_{0·63}$
2·5	$4·0 \times 10^{-3} \times TD_{0·63}$
3·5	$1·9 \times 10^{-2} \times TD_{0·63}$
4·5	$4·6 \times 10^{-2} \times TD_{0·63}$
5·5	$8·1 \times 10^{-2} \times TD_{0·63}$
6·5	$1·2 \times 10^{-1} \times TD_{0·63}$

$TD_{0·63}$ = Dose that produces a tumour rate of 0·63.

Agency, 1977; National Academy of Sciences, 1977) where the same slides were read by many different pathologists with widely varying results). The rule to be followed here for the liver of the mouse is to take as positive any response that the author of the article declares malignant (hepatocellular carcinoma) or implies is a precursor (hepatoma, nodule, neoplasm etc.). To the extent that it is possible, the Weibull model will be fitted to both endpoints (any tumour or malignant tumour) with the mouse liver. This rule does not apply to the liver of the rat, where a carcinoma is taken as the endpoint.

There are 31 studies included here. These are described briefly in the first seven columns of Table 2. To illustrate, take the first row (study 1) for 2-acetylaminofluorene (2-AAF). This is an ingestion experiment in the female mouse (BALB/c strain), and a liver tumour is the endpoint. The actual experimental results are given in Table 3. This is, of course, the largest experiment ever run (3383 animals in the 24-month kill). Study 14 here is the same experiment, but with liver carcinoma as the endpoint. In this same experiment, there were serial kills (after 9, 12, 14, 15, 16, 17, 18, 24 or 33 months of treatment) producing another dose-response function (actually, one for each dose group), listed here as study 13 for age at kill as the cause of the liver tumours.

Study 27 requires a special comment. Here the pathological endpoint is a chromophobe adenoma of the pituitary. While this may not be a malignant tumour, it is a life-threatening brain tumour, and the study is included for this reason. The implied cause of the tumour is also interesting. The 'dose' (see Table 3) is the body weight at 70 days of age. The rats were fed a range of protein levels and caloric contents, which produced the various body weights.

Studies 29, 30 and 31 for 2-AAF (again), asbestos and bis(chloromethyl)ether are not true lifetime exposure studies. In each of these, only one dose of the agent was used, but for various fractions of the animals' lifetimes. This is actually a completely different dimension to the dose-response situation. These three studies are included for the following reasons: these agents are important; this dimension to the situation

Table 2. *Results of fitting the Weibull model to the observed data*

Study no.	Agent*	Route	Species and strain	Sex	Endpoint	*P*	Est. m	SE m	I/2†
1	2-AAF	Diet	Mo BALB c	f	Liver T	0·56	1·49	0·11	3/2
2	DDT	Diet	Mo CF 1	m	Liver T	0·19	0·33	0·083	1/2
3	DDT	Diet	Mo CF 1	f	Liver T	0·08	1·44	0·31	3/2
4	HEOD	Diet	Mo CF 1	m	Liver T	0·99	2·10	0·49	3/2, 5/2
5	HEOD	Diet	Mo CF 1	f	Liver T	0·32	1·41	0·49	3/2
6	Hept.	Diet	Mo B6C3F1 CD1	m f	Liver T	0·87	3·51	?	7/2
7	Chlor.	Diet	Mo B6C3F1 CD1	m f	Liver T	0·49	4·50	?	9/2
8	Tox.	Diet	Mo B6C3F1	m	Liver T	0·71	1·71	0·28	3/2
9	Tox.	Diet	Mo B6C3F1	f	Liver T	0·92	1·89	0·30	3/2
10	Gris.	Diet	Mo Swiss	m f	Liver T	0·10	1·56	0·24	3/2
11	HS	po	Mo CBA	m	Liver T	0·08	2·30	0·91	5/2
12	HS	po	Mo CBA	f	Liver T	0·65	4·24	1·47	9/2
13	Age‡		Mo BALB c	f	Liver T		6·50		
14	2-AAF	Diet	Mo BALB c	f	Liver C	0·09	1·73	0·14	3/2
15	HEOD	Diet	Mo CF 1	m f	Liver C	0·38	1·49	0·26	3/2
16	Tox.	Diet	Mo B6C3F1	m	Liver C	0·47	2·04	0·34	3/2, 5/2
17	Tox.	Diet	Mo B6C3F1	f	Liver C	0·54	3·64	0·81	7/2
18	DMN	Diet	Mo RF	m	Liver A	0·04	2·64	0·35	5/2
19	2-AAF	Diet	Mo BALB c	f	Bladder C	0·15	6·46	0·43	13/2
20	DES	Diet	Mo C3H	m	Mammary C	0·70	1·34	0·22	3/2
21	DES	Diet	Mo A	m(c)	Mammary C	0·84	0·64	0·12	1/2
22	DES	Diet	Mo C3H	f	Mammary C	0·65	0·51	0·081	1/2
23	Afla.	Diet	R Fischer	m	Liver C	0·64	1·58	0·29	3/2
24	Afla.	Diet	R Fischer	m	Liver C	0·42	1·49	0·18	3/2
25	DMN	Diet	R Porton	f	Liver C	0·24	1·43	0·24	3/2
26	VC	ih	R Sprague–Dawley	m f	Liver A	0·50	0·48	0·12	1/2
27	Diet§	Diet	R Charles River	m	Pituitary T	0·41	1·34	0·21	3/2
28	ETU	Diet	R Charles River	m f	Thyroid C	0·77	3·33	0·42	7/2
29	2-AAF	Diet	Mo BALB c	f	Bladder C	0·86	2·23	0·34	
30	Asbes.	ih	R Wistar	m f	Lung C	0·17	1·20	0·17	
31	BCME	ih	R Sprague–Dawley	m	Resp. ca.	0·79	1·83	0·43	

po = Oral intubation ih = Inhalation Mo = Mouse R = Rat c = Castrated T = Tumour C = Carcinoma A = Angiosarcoma Resp. ca. = Respiratory cancer *P* = The *P* value from the chi-square test for goodness of fit (a large *P* value, near 1·0, indicates a good fit, and a small *P* value, near 0, indicates a poor fit). Est. m = The statistical best estimate of the shape parameter in the Weibull model by weighted least squares. SE m = The approximate standard error for the best estimate of m.

*See Table 3 for full names of agents.

†The suggested value of m under the hypothesis that the 'true' shape parameter is of the form I/2, where I is a positive (odd) integer.

‡The animals were serially killed after 9, 12, 14, 15, 16, 17, 18, 24 or 33 months of treatment.

§Controlled variations in the diet (calories and proteins) produced variations in body weight at 70 days of life.

is important (for human occupational exposure); and abundant data were produced.

Now turn to the application of the Weibull model to each of the thirty-one studies listed in Table 2. (The statistical details are illustrated in the Appendix.) Each *P*-value given in the seventh column of Table 2 gives a measure of the goodness of the fit of the Weibull model to the data for that study. A large *P*-value (near 1·00) indicates a good fit, and a small *P*-value (near 0) indicates a poor fit.

The distribution of the *P*-values in Table 2 is favourable to the hypothesis that the Weibull model

Table 3. *Observed experimental results*

	Observed results								Calculated Weibull model parameters
Study 1. 2-Acetylaminofluorene (2-AAF), 24-month kill[1]									
Dose (ppm)	0	30	35	45	60	75	100	150	m = *1·49*
No. at risk	383	900	639	445	415	311	160	130	α = *0·0209*
Observed no. of TBA	9	55	55	57	71	62	47	56	β = *0·000333*
Calculated no. of TBA	7·9	64·0	53·5	49·4	64·7	63·4	46·0	58·9	
Study 2. DDT[2]									
Dose (ppm)	0	2	10	50	250				m = *0·33*
No. at risk	58	66	51	74	53				α = *0·269*
Observed no. of TBA	13	32	24	43	44				β = *0·199*
Calculated no. of TBA	13·7	26·7	25·5	46·6	41·2				
Study 3. DDT[2]									
Dose (ppm)	0	2	10	50	250				m = *1·44*
No. at risk	55	49	65	49	41				α = *0·0353*
Observed no. of TBA	2	1	9	6	29				β = *0·000414*
Calculated no. of TBA	1·9	1·7	3·0	6·9	28·8				
Study 4. Dieldrin (HEOD)[3]									
Dose (ppm)	0	1·25	2·5	5	10*	20*			m = *2·10*
No. at risk	78	30	30	30	11	17			α = *0·122*
Observed no. of TBA	9	6	13	26	5	12			β = *0·0646*
Calculated no. of TBA	9·1	6·1	12·9	26·0					
Study 5. Dieldrin (HEOD)[3]									
Dose (ppm)	0	1·25	2·5	5	10*	20*			m = *1·41*
No. at risk	78	30	28	30	17	21			α = *0·104*
Observed no. of TBA	8	5	12	18	9	8			β = *0·0887*
Calculated no. of TBA	7·7	6·2	9·7	18·5					
Study 6A. Heptachlor (B6C3F1, male)[4]									
Dose (ppm)	0	6·1	13·8						m = *3·51*
No. at risk	19	45	45						α = *0·333*
Observed no. of TBA	5	14	24						β = *0·0000431*
Calculated no. of TBA	5·4	13·5	24·0						
Study 6B. Heptachlor (CD 1 male)[4]									
Dose (ppm)	0	1	5	10					m = *3·51*
No. at risk	59	58	66	73					α = *0·0232*
Observed no. of TBA	2	1	4	37					β = *0·000136*
Calculated no. of TBA	1·3	1·3	4·0	27·0					
Study 6C. Heptachlor (B6C3F1 female)[4]									
Dose (ppm)	0	9	18						m = *3·51*
No. at risk	10	44	42						α = *0·0265*
Observed no. of TBA	1	3	21						β = *0·0000255*
Calculated no. of TBA	0·3	3·3	20·7						
Study 6D. Heptachlor (CD 1 female)[4]									
Dose (ppm)	0	1	5	10					m = *3·51*
No. at risk	74	71	65	52					α = *0·00543*
Observed no. of TBA	1	0	3	16					β = *0·000115*
Calculated no. of TBA	0·4	0·4	2·4	16·3					
Study 7A. Chlordane (B6C3F1 male)[4]									
Dose (ppm)	0	29·9	56·2						m = *4·50*
No. at risk	20	45	46						α = *0·249*
Observed no. of TBA	5	16	30						$\beta = 9{\cdot}57 \times 10^{-9}$
Calculated no. of TBA	5·9	14·6	30·1						

Table 3. (*continued*)

	Observed results							Calculated Weibull model parameters
Study 7B. Chlordane (CD 1 male)[4]								
Dose (ppm)	0	5	25	50*				m = *4·50*
No. at risk	33	55	51	44				α = *0·174*
Observed no. of TBA	4	11	30	25				$\beta = 3{\cdot}65 \times 10^{-7}$
Calculated no. of TBA	5·3	8·8	30·0					
Study 7C. Chlordane (B6C3F1 female)[4]								
Dose (ppm)	0	30·1	63·8					m = *4·50*
No. at risk	19	46	47					α = *0·0340*
Observed no. of TBA	1	2	20					$\beta = 3{\cdot}90 \times 10^{-9}$
Calculated no. of TBA	0·6	2·3	19·9					
Study 7D. Chlordane (CD 1 female)[4]								
Dose (ppm)	0	5	25	50*				m = *4·50*
No. at risk	44	61	51	40				α = *0·00436*
Observed no. of TBA	1	0	23	22				$\beta = 3{\cdot}05 \times 10^{-7}$
Calculated no. of TBA	0·2	0·3	23·0					
Study 8. Toxaphene[5]								
Dose (ppm, TWA)	0	5·04	14·4	36·0	82·5	167		m = *1·71*
No. at risk	63	54	53	51	49	46		α = *0·197*
Observed no. of TBA	12	10	12	18	40	45		β = *0·00065*
Calculated no. of TBA	11·2	10·1	12·1	19·9	37·2	45·4		
Study 9. Toxaphene[5]								
Dose (ppm, TWA)	0	5·04	14·4	36·0	82·5	167		m = *1·89*
No. at risk	62	53	52	52	49	49		α = *0·0383*
Observed no. of TBA	2	2	4	6	18	40		β = *0·000103*
Calculated no. of TBA	2·3	2·1	2·7	6·3	18·4	39·8		
Study 10. Griseofulvin[6]								
Dose (%)	0	0·3	1·5	3·0				m = *1·56*
No. at risk	196	53	57	41				α = *0*
Observed no. of TBA	0	2	35	35				β = *0·3923*
Calculated no. of TBA	0	3·1	29·8	36·4				
Study 11. Hydrazine sulphate (HS)[7]								
Dose (mg)	0	0·14	0·28	0·56	1·13*			m = *2·30*
No. at risk	30	26	25	25	25			α = *0·0493*
Observed no. of TBA	3	1	7	12	15			β = *2·429*
Calculated no. of TBA	1·4	1·9	4·1	12·4				
Study 12. Hydrazine sulphate (HS)[7]								
Dose (mg)	0	0·14	0·28	0·56	1·13*			m = *4·24*
No. at risk	29	25	25	24	24			α = *0·0220*
Observed no. of TBA	1	0	2	16	15			β = *12·61*
Calculated no. of TBA	0·6	0·6	1·9	16·0				
Study 14. 2-Acetylaminofluorene (2-AAF), 24-month kill[8]								
Dose (ppm)	0	30	35	45	60	75	100	150	m = *1·73*
No. at risk	383	900	639	445	415	311	160	130	α = *0·01168*
Observed no. of TBA	7	24	27	36	47	42	27	42	β = *0·0000694*
Calculated no. of TBA	4·4	32·4	27·7	26·7	37·4	38·8	30·6	44·2	
Study 15. Dieldrin (HEOD)[3]								
Dose (ppm)	0	1·25	2·5	5·0	10·0*	20·0*		m = *1·49*
No. at risk	156	60	58	60	28	38		α = *0·0003*
Observed no. of TBA	0	2	2	8	3	12		β = *0·0121*
Calculated no. of TBA	0	1·0	2·7	7·5				
Study 16. Toxaphene[5]								
Dose (ppm)	0	5·04	14·4	36·0	82·5	167		m = *2·05*
No. at risk	63	54	53	51	49	46		α = *0·1507*
Observed no. of TBA	7	10	11	12	34	45		β = *0·0001143*
Calculated no. of TBA	8·8	7·7	8·6	14·0	32·3	45·2		

Table 3. (*continued*)

	Observed results							Calculated Weibull model parameters
Study 17. Toxaphene[5]								
Dose (ppm)	0	5·04	14·4	36·0	82·5	167		$m = 3{\cdot}64$
No. at risk	62	53	52	52	49	49		$\alpha = 0{\cdot}02539$
Observed no. of TBA	1	1	3	3	5	34		$\beta = 9{\cdot}416 \times 10^{-9}$
Calculated no. of TBA	1·5	1·3	1·3	1·5	5·3	33·9		
Study 18. Dimethylnitrosamine (DMN)[9]								
Dose (mg/kg)	0	0·20	0·43	0·91				$m = 2{\cdot}64$
No. at risk	120	17	47	93				$\alpha = 0{\cdot}00621$
Observed no. of TBA	1	0	24	89				$\beta = 4{\cdot}270$
Calculated no. of TBA	0·7	1·1	17·5	89·7				
Study 19. 2-Acetylaminofluorene (2-AAF), 24-month sacrifice[1]								
Dose (ppm)	0	30	35	45	60	75	100	150
No. at risk	384	900	638	445	415	311	160	130
Observed no. of TBA	1	0	2	1	3	3	25	100
Calculated no. of TBA	0·2	0·5	0·4	0·5	1·9	5·4	16·6	101·1
								$m = 6{\cdot}46$
								$\alpha = 0{\cdot}000458$
								$\beta = 1{\cdot}32 \times 10^{-14}$
Study 20. Diethylstilboestrol (DES)[10]								
Dose (ppm)	0	6·25	12·5	25	50	100	500	1000*
No. at risk	115	59	58	62	62	60	60	71
Observed no. of TBA	0	0	1	0	3	3	23	30
Calculated no. of TBA	0	0·1	0·2	0·5	1·4	3·3	23	
								$m = 1{\cdot}34$
								$\alpha = 0$
								$\beta = 0{\cdot}000117$
Study 21. Diethylstilboestrol (DES)[10]								
Dose (ppm)	0	6·25	12·5	25	50	100	500	1000
No. at risk	136	78	78	70	77	74	52	76
Observed no. of TBA	0	0	1	2	3	6	7	15
Calculated no. of TBA	0	0·7	1·1	1·5	2·6	3·8	7·2	15·7
								$m = 0{\cdot}64$
								$\alpha = 0$
								$\beta = 0{\cdot}00279$
Study 22. Diethylstilboestrol (DES)[10]								
Dose (ppm)	0	6·25	12·5	25	50	100	500	1000
No. at risk	121	56	60	60	68	64	59	58
Observed no. of TBA	40	27	26	26	36	42	50	50
Calculated no. of TBA	40·1	23·3	26·8	29·3	37·0	39·2	47·6	5·14
								$m = 0{\cdot}51$
								$\alpha = 0{\cdot}403$
								$\beta = 0{\cdot}0521$
Study 23. Aflatoxin B_1[11]								
Dose (ppb)	0	1	5	15	50	100		
No. at risk	18	22	22	21	25	28		$m = 1{\cdot}58$
Observed no. of TBA	0	2	1	4	20	28		$\alpha = 0{\cdot}0178$
Calculated no. of TBA	0·3	0·5	1·2	4·3	19·1	27·6		$\beta = 0{\cdot}00297$
Study 24. Aflatoxin B_1[12]								
Dose (ppb)	0	5	15	50	200			
No. at risk	64	60	63	63	64			$m = 1{\cdot}49$
Observed no. of TBA	1	1	2	18	55			$\alpha = 0{\cdot}00891$
Calculated no. of TBA	0·6	1·0	3·2	14·6	55·5			$\beta = 0{\cdot}000751$
Study 25. Dimethylnitrosamine (DMN)[13]								
Dose (ppm)	0	2	5	10	20	50		
No. at risk	29	18	62	5	23	12		$m = 1{\cdot}43$
Observed no. of TBA	0	0	4	2	15	10		$\alpha = 0$
Calculated no. of TBA	0	0·4	4·9	1·0	10·4	10·7		$\beta = 0{\cdot}00828$
Study 26. Vinyl chloride (VC)[14]								
Dose (ppm)	0	50	250	500	2500	6000	10000*	
No. at risk	58	59	59	59	59	60	61	$m = 0{\cdot}48$
Observed no. of TBA	0	1	4	7	13	13	9	$\alpha = 0$
Calculated no. of TBA	0	1·7	3·6	5·0	10·3	15·1		$\beta = 0{\cdot}00447$
Study 27. Diet[15]								
Dose (g)†	64	78	80	143	301	320		
No. at risk	352	250	250	250	250	250		$m = 1{\cdot}34$
Observed no. of TBA	3	3	6	14	20	26		$\alpha = 0$ (assumed)
Calculated no. of TBA	4·2	3·9	4·0	8·7	22·8	24·6		$\beta = 0{\cdot}0000455$

Table 3. (*continued*)

Observed parameters			Observed results				Calculated Weibull model parameters
Study 28. Ethylene thiourea (ETU)[16]							
Dose (ppm)	0	5	25	125	250	500	m = *3·33*
No. at risk	72	75	73	73	69	70	α = *0·0182*
Observed no. of TBA	2	2	1	2	16	62	β = *2·25 × 10^{-9}*
Calculated no. of TBA	1·3	1·4	1·3	2·9	14·5	62·3	
Study 29. 2-Acetylaminofluorene (2-AAF), 24-month kill[1]							
Dose‡	0	0·375	0·500	0·625	1·0		m = *2·23*
No. at risk	384	33	29	28	130		α = *0·00262*
Observed no. of TBA	1	6	7	11	100		β = *1·455*
Calculated no. of TBA	1·0	5·0	7·8	11·2	99·7		
Study 30. Asbestos[17]							
Dose§	0	1/30	3	6	12	24	m = *1·20*
No. at risk	126	219	180	90	129	95	α = *0·0042*
Observed no. of TBA	0	3	8	7	35	37	β = *0·0118*
Calculated no. of TBA	0·5	1·0	8·5	9·0	27·1	39·5	
Study 31. Bis(chloromethyl)ether (BCME)[18]							
Dose‖	10	20	40	60	80	100	m = *1·83*
No. at risk	41	46	18	18	34	20	α = *0·0157*
Observed no. of TBA	1	3	4	4	15	12	β = *0·000183*
Calculated no. of TBA	1·1	2·7	2·8	5·2	14·8	11·5	

TBA = Tumour-bearing animals TWA = Time-weighted average

[1]Staffa & Mehlman (1979); [2]Tomatis, Turusov, Day & Charles (1972); [3]Walker, Thorpe & Stevenson (1972); [4]National Academy of Sciences (1977); [5]National Cancer Institute (1977) & Litton Bionetics (1978); [6]Rustia & Shubik (1978); [7]Biancifiori (1970); [8]National Centre for Toxicological Research, personal communication (1980); Clapp and Toya (1970—the lowest dose level, 0·20 is an estimate); [10]Gass, Coats & Graham (1964); [11]Wogan, Paglialunga & Newberne (1974); [12]WARF Institute, Inc. (Groups 1 & 3) (c. 1975); [13]Terracini, Magee & Barnes (1967); [14]Maltoni (1976); [15]Ross, Bras & Ragbeer (1970); [16]Graham, Davis, Hansen & Graham (1975); [17]Wagner, Berry, Skidmore & Timbrell (1974); [18]Kuschner, Laskin, Drew, Cappiello & Nelson (1975).

*An asterisk indicates a datum omitted because of reported excessive toxicity and/or an apparent break in the dose-response function. In study 20 dropping the top dose is questionable. With all eight doses included, the estimated m is 1·03 (SEM = 0·14) and the *P* value is 0·28.

†Body weight at 70 days of age.

‡Months of exposure out of 24.

§Months of exposure.

‖Number of exposures.

describes the dose-response functions for carcinogenesis. Under this hypothesis, one would expect a uniform distribution of *P*-values over the interval 0 to 1, and that certainly has occurred. Of course, this does not prove that the Weibull model is the one 'true' model.

The eighth and ninth columns of Table 2 give the estimated value of the parameter m and the approximate standard error of this estimate for each study. Approximate confidence intervals for the true value of the parameter m can be constructed with these two numbers. For example, take the first row for Study 1 with 2-AAF. The best estimate of the value of m is 1·49, and a 95% confidence interval for this is $1{\cdot}49 \pm (2 \times 0{\cdot}11)$ or 1·27 to 1·71.

Next consider the one-hit model, which is a special case of the Weibull model (with m = 1). This has been widely proposed as the correct model for carcinogenesis. The most surprising thing in Table 2 is that none of the estimated values of m is close to 1·00 (relative to its standard error). In fact, the data for almost all of these studies reject the one-hit model in the statistical sense.

Finally, there is a very interesting pattern among the estimated values of m in Table 2. Notice that each of these tends to be numerically close to a fraction with an odd integer in its numerator and a '2' in its denominator (using the standard error to measure 'close'). These nearest fractions are given in the tenth column labelled 'I/2'. The numerators alone (the I-values) then suggest a very curious hypothesis: the dose-response functions for carcinogenesis tend to fall into categories each of which is associated with an odd positive integer. When this odd positive integer is divided by two, the resulting fraction is the value of the shape parameter (m) in the Weibull model describing the dose-response function.

A further implication from these categories is that there might be some physiological factor which has a constant value in one category and which varies from

category to category in some way that is correlated with the category integer.

Further research in this direction would encounter the problem of negative studies. For example, take DDT which produced liver tumours in both sexes of the CF-1 mouse as shown in studies 2 and 3. However, the National Cancer Institute tested DDT using the B6C3F1 mouse and failed to find tumours (or any others) in either sex (National Cancer Institute, 1978). This could mean that DDT is not tumorigenic in this strain or that DDT is tumorigenic in this strain, but the category of the dose-response function has a high integer (like I = 5). Under this assumption, the NCI experiment failed to reveal the tumorigencity because the toxicity of DDT prevented the use of high enough doses. In other words, the maximum tolerated dose is below the steep part of the curve for this strain. (During the experiment, the NCI varied the levels of the high-dose group, depending on observed weight gain and mortality, in an attempt to reach the maximum tolerated dose. Their final reported dose is actually a time-weighed average).

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APPENDIX

The simple form of the Weibull model is:

$$P = 1 - e^{-(\alpha + \beta x^m)},$$

where x is the dose, P is the tumour rate and m, α, β are parameters to be estimated from the data. At very low doses, the excess risk over background is (approximately) βx^m. That is, the risk is proportional to the m-th power of the dose. The virtual safe dose (VSD) corresponding to a one-in-a-million risk over background is then given by

$$VSD = (10^{-6}/\beta)^{1/m}.$$

Weighted least squares offers one method of estimating the parameters in the model from a data set. This method was used in obtaining the statistical results in Table 2. The first step is to partially linearize the model by the transformation

$$Y = -\ln(1 - P) = \alpha + \beta x^m.$$

The weight (W) for an observed value of Y is given by $W = nQ/P$, where $Q = 1 - P$ and n = the number of animals at risk. With only a linear weighted least-squares program, trial and error on the parameter m is necessary. (A convenient program for a TI-59 calculator for this method is available from the author).

The Weibull model can be generalized to include a simultaneous analysis of the results from more than one experiment which shares a common value of the parameter m:

$$P = 1 - e^{(\alpha_i + \beta_i x^m)},$$

where i is an index for the subexperiments. Study 6 here for heptachlor (and Study 7 for chlordane) illustrates this feature. No one of the four substudies (6A, 6B, 6C, 6D) contains two responses clearly greater than the tumour rate for its control group. But all four together form one large study, on the assumption that they all share a common value of the parameter m.

Another interesting generalization of the Weibull model includes the age of the animals when killed (t):

$$P = 1 - e^{(\alpha + \beta x^m)t^k},$$

where k is a new parameter. The large experiment with 2-AAF included many early kills in addition to the major kill at 24 months. With a liver tumour as the endpoint (see p. 26 of Staffa & Mehlman, 1979), this version of the Weibull model fits these data with the following estimates:

$m = 1{\cdot}45$ (to the nearest 0·05) and $k = 6{\cdot}50$ (to the nearest 0·25). This is the analysis which led to the line in Table 2 for Study 13.

One of the most important empirical discoveries in this entire field is that of Druckrey and his colleagues (Druckrey, 1967). As the following derivation shows, Druckrey's result is implied by the Weibull model. That is, it is a corollary of the Weibull model for carcinogenesis.

As stated previously, an extended form of the Weibull model is:

$$P = 1 - e^{-(\alpha + \beta x^m)t^k}.$$

Now suppose that t measures the time to a tumour (rather than the time to sacrifice). Also, suppose that the background tumour rate is zero ($\alpha = 0$). In much of Druckrey's work, the tumours were palpable, life-terminating liver cancers in rats where the background rate is essentially zero. (Many others were skin-painting studies, again with a background rate of zero). For a test group at some dose x, consider the median time to tumour—that is, the value of t such that $P = 0{\cdot}50$. The extended Weibull model for this dose and time is

$$0{\cdot}50 = 1 - e^{-\beta x^m t^k}.$$

This reduces to $[-(\ln. 50/\beta)]^{1/n} = x\,t^{k/m} = x\,t^n$, where $n = k/m$ and the left side of the equation is a constant. But this is exactly Druckrey's result: the dose times the median time to a tumour raised to a power is a constant.

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REVIEWS OF RECENT PUBLICATIONS

World Review of Nutrition and Dietetics. Vol. 29. **Toxicology and Nutrition.** Edited by R. Truhaut & R. Ferrando. S. Karger AG. Basel, 1978. pp. x + 190. DM 119.00.

This volume in the well established, if somewhat grandly titled, series records the proceedings, although not the associated discussions, of what is claimed to be the first international symposium specifically designed to bring together the areas of nutrition and toxicology. The symposium was held at Alfort, France at the end of 1976 and attracted a number of eminent biochemists with interests in both of these areas.

The outcome of this initiative is a somewhat disjointed mixture of quite lengthy general reviews and shorter papers on specific topics, the majority of which are, however, interesting, informative and of a consistently high standard. The symposium is introduced by Raymond Jacquot (Paris) with a plea for researchers in nutrition and toxicology to get together to find a new discipline—metabolic toxicology. This is followed by a lengthy review by the editors emphasizing the close relationship between toxicology and nutrition. Somogyi (Zurich) reviews some of the naturally occurring toxic compounds found in foods, such as those causing favism (pyrimidine glycosides in broad beans) and lathyrism (nitriles in vetch) and various antivitamins. Later in the volume the pharmacodynamic action of a wide range of naturally occurring food chemicals is discussed by Sapieka (South Africa) who suggests that many as yet unrecognized compounds may produce subtle toxic effects by this mechanism. The role and importance of the gastro-intestinal microorganisms in the biotransformation of orally ingested xenobiotics is comprehensively reviewed by R. V. Smith (Texas) and the toxicological consequences of microbial reactions (predominantly reductive) are stressed. Lechat (Paris) deals in a general way with other reactions that foreign compounds can undergo in the body with particular reference to activation processes leading to the production of toxic intermediates. The complex problem of the interaction between drugs, toxins and nutrients is reviewed at some length by Newberne and coworkers (New York) and the importance of an interdisciplinary approach is highlighted.

In addition to the reviews, contributions on more specific areas are included. Thus Parke (Surrey) discusses the consequences of long-term drug administration on the requirement for ascorbic acid and folic acid, McLean (London) discusses some of his work on nutrition and hepatic injury produced by paracetamol and Garner (York) discusses his work on diet and the biological effects of aflatoxin. Finally Ganzin (FAO) tackles the very difficult area of risk evaluation.

Overall the volume is well produced. It provides a valuable source of information both for toxicologists with nutritional leanings and for nutritionists with an interest in toxicology.

The HLA System. An Introductory Survey. 2nd Revised Ed. Edited by A. Svejgaard, M. Hauge, C. Jersild, P. Platz, L. P. Ryder, L. Staub Nielsen & M. Thomsen. S. Karger AG. Basel, 1979. pp. viii + 111. Sw.fr. 36.00.

Awareness of the HLA system developed out of a search for blood groups of leucocytes that could form the basis for matching donors and recipients for transplantation. Subsequently, mainly through experimental work on inbred strains of mice, it has been shown to be a complex genetic system controlling cell-surface determinants involved in cellular interactions in the immune system. Interest in these genetically determined antigens has been greatly increased by the demonstration that not only are certain HLA antigens associated with various diseases, but that there are large individual variations in host susceptibility due to genetic factors. It is naturally a great challenge for immunologists and geneticists to clarify the biological functions of HLA in man or related systems in animals.

This monograph provides a most helpful introduction to the HLA system with brief notes on nomenclature and a chapter on the components of the system. This latter chapter also includes a discussion on the function of immune response (Ir) genes and the close association between HLA and certain complement components. There is an interesting discussion on the high polymorphism of HLA antigens which could be due to selective pressures in the past, perhaps operating through resistance to diseases from which we are now protected. The role of HLA in clinical medicine is described as well as the population and family studies undertaken to investigate the genetics of disease. There is also an appendix on methodology which includes both serological methods for HLA typing and mixed-lymphocyte assays.

The introductory monograph is excellent background for the scientist interested in studying host susceptibility and responsiveness to various environmental agents.

Immunopathology. Sixth International Convocation on Immunology. Edited by F. Milgrom & B. Albini. S. Karger AG, Basel, 1979. pp. x + 350. Sw.fr. 149.00.

The field of immunopathology has evolved with the increasing knowledge that immunological mechanisms quite frequently bring more harm than benefit, often as an exaggeration of a normal physiological mechanism. This book summarizes the proceedings of the Sixth International Convocation on Immunology, held in Niagara Falls, 12–15 June 1978. The subject of the Convocation was immunopathology with special emphasis on autoimmunity.

The formation of autoantibodies, that is antibodies combining with autologous ('self') antigens under natural and experimental conditions, are described and discussed by a number of contributors. Whilst the immune system normally does not produce antibodies directed against components of the same organism, since lymphocytes possessing receptors for self antigens normally exist in the resting state or are suppressed, autoimmune reactions can be induced by antigenic alteration of the body's own constituents by viral infections or by changes in the immunoregulatory mechanism. One section of the book is devoted to immunologically mediated organ-restricted diseases and particularly to autoimmune thyroid disease and to renal injury due to immune complexes. It appears that the renal interstitium is a site where cell-mediated reactions can occur and that autologous or exogenous antigens have been implicated in interstitial nephritis in both experimental animals and man. Delayed hypersensitivity reactions may play a role in nephritis that develops after administration of certain drugs.

Several chapters deal with immunologically mediated systemic diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis and serum sickness, and others cover the immunopathology of infectious diseases. SLE has become the prototype of autoimmune pathology, and much of the tissue damage is due to the presence of antinuclear antibodies. It is of interest that SLE may be of the idiopathic type (genetically based) or drug-induced, and that the New Zealand Black mouse model can be used to study some aspects of this disease.

The last section of the book deals with effector mechanisms in immunopathology, and includes discussions of the association of immune complex disease with complement factors, the crucial role of IgE antibody in reaginic hypersensitivity and atopic diseases, and the Hageman factor system. This latter system of plasma proteins can cause a significant number of inflammatory changes including increased vascular permeability, smooth muscle contraction and hypotension.

This book is most informative and has achieved a good balance between authoritative reviews of well-established concepts, reports on recent data and hypothesis.

Mass Spectrometry. Vol. 5. **A Review of the Recent Literature Published between July 1976 and June 1978.** Senior Reporter R. A. W. Johnstone. The Chemical Society, London, 1979. pp. xii + 450. £25.00.

This volume in the 'Specialist Periodical Report' series brings the coverage of the literature on the theory, techniques and applications of mass spectrometry up to June 1978. By way of decennial celebration a cumulative subject index has been included for the first time in the mass spectrometry reports. Although a major weakness of previous volumes is thus partly repaired, the inclusion of indexes in each volume would have been more valuable.

The chapters of this volume range, as usual, over the theoretical and the practical, the esoteric and the popular, the established and the novel. Developments in the theory and energetics of ion chemistry, and in the investigation of the relationship between structure and the energetics and mechanisms of ion reactions are covered extensively in the first two chapters. Four further chapters are devoted to progress in selected techniques, namely, photoelectron–photoion coincidence spectroscopy, computer-assisted data acquisition and interpretation, gas chromatography–mass spectrometry and instrumentation trends generally. Seven chapters on applications in the fields of drug metabolism, food science, the environment, organic geochemistry, fragmentation studies, natural-product chemistry, and organometallic-, co-ordination- and inorganic-compound chemistry, complete this well-balanced and most valuable addition to an invaluable series.

Editing Scientific Books and Journals. An ELSE–Ciba Foundation Guide for Editors. By M. O'Connor. Pitman Medical Publishing Co. Ltd., Tunbridge Wells, 1978. pp. vi + 218. £7.00.

The advice in this guide for editors could save us all a lot of time spent reading between the lines. It is a useful book for anyone with an interest in scientific publishing as well as for editors. It introduces the processes of publication and describes the many facets of editing multi-author books, conference proceedings and journals. The responsibilities of editors towards readers, authors and science itself are emphasized. The author provides insight into the decisions that an editor must make and in many of these the editor must have his mind clearly focussed on the reader. This book should also help those faced with the task of writing scientific manuscripts to get the best from their editors and publishers.

The chapters covering the main stages of publishing apply to all three types of publication. Of the three, conference proceedings are perhaps the most dangerous zone for editors. Such publications are frequently criticized on many counts; amongst these are that they repeat data already published in journals and that they are carelessly edited. A separate chapter discusses the problems of publishing conference proceedings. Clearly the potential publication should be a major consideration in the planning stages of the conference. The author emphasizes that some worthwhile conferences do not make worthwhile publications.

Chapters on the financial aspects of starting a new journal and editing a successful journal are particularly interesting and offer some sound advice. Separate appendices include suggested guidelines for authors, referees and book reviewers and suggestions about text citations and reference lists. This book as a whole is liberally scattered with references and the reference list should prove useful to anyone interested in scientific publishing.

BOOKS RECEIVED FOR REVIEW

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Selected Methods in Cellular Immunology. Edited by B. B. Mishell & S. M. Shiigi. W. H. Freeman & Co., Oxford, 1980. pp. xxix + 486. £17.70.

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The Science of 2,4,5-T and Associated Phenoxy Herbicides. By R. W. Bovey & A. L. Young. John Wiley & Sons Ltd., Chichester, 1980. pp. ix + 462. £20.50.

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Experiments with Normal and Transformed Cells. A laboratory manual for working with cells in culture. By R. Crowe, H. Ozer & D. Rifkin. Cold Spring Harbor Laboratory, New York, 1978. pp. 175. $19.80.

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Progress in Environmental Mutagenesis. Edited by M. Alacević. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. x + 347. Dfl. 102.00.

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Information Section

ARTICLES OF GENERAL INTEREST

ASSESSMENT OF A MERCAPTO-FUNCTIONAL SILICONE OIL

The polydimethylsiloxanes are effective antifoam agents and are used in food processing, in drug and cosmetic preparations, and in some types of plastic surgery. They have caused tissue reactions in some prosthetic applications (*Cited in F.C.T.* 1966, **4,** 190), but otherwise are of very low toxicity (*ibid* 1967, **5,** 263; Cutler *et al. Fd Cosmet. Toxicol.* 1974, **12,** 443; *cited in F.C.T.* 1977, **15,** 79). However, little has been known hitherto about the toxicity of certain polydimethylsiloxane derivatives that contain a mercapto group and have been developed for industrial use. Into this gap has now come a series of papers describing a range of studies on a mercapto-polydimethylsiloxane in which a methyl group is replaced at intervals by a γ-mercaptopropyl group to give an oil with a low volatility (<0·5% at 400°C), a viscosity of 250–300 cS and a sulphur content of about 0·08%. This oil has been developed for use as a release agent in xerographic copying processes.

In acute inhalation tests on this mercapto-functional silicone oil (MSO), young rats were exposed to vapour generated by heating the oil at 150 or 180°C (Parent, *Drug Chem. Toxicol.* 1979, **2,** 295). There were no deaths or behavioural effects during inhalation of an atmosphere containing approximately 1·1 g MSO/m^3 air or during the 14 days following the exposure (the duration of which is not stated). A decrease in weight gain to about 40% of the gain in the controls occurred in the rats exposed to vapour generated at 180°C. This effect was less marked with vapour generated at the lower temperature. No clear effects on weight gain over a 14-day observation period were found by Parent (*loc. cit.*) in rats dosed orally, after fasting, with a single dose of 10·3, 15·4, 23·1 or 34·6 g MSO/kg. Again there were no deaths, but hypoactivity, ruffled fur and laboured breathing were noted within 30 min of treatment, and diuresis occurred 6–22 hr after administration of the two highest doses. Autopsy of the animals exposed to MSO vapour revealed no gross pathological effects but the rats treated orally showed inflammation of the stomach and intestines 14 days after treatment. (It is not entirely clear from the paper whether inflammation occurred in all of the treated animals, or only in those given high doses of MSO.)

Parent (*loc. cit.*) continued his report of acute studies with the results of irritancy and sensitization tests in the rabbit, guinea-pig and man. Application of undiluted MSO (in a dose of 3 g/kg, over about 30% of the total body surface) to the shaved, scarified skin of rabbits, with plastic-sheet occlusion for 24 hr, produced no deaths or behavioural effects. There was barely perceptible erythema after 24 hr but no skin reactions were apparent at days 7 and 14, and no gross pathological changes were seen at autopsy after 14 days. The dermal LD_{50} thus exceeded the test dose of 3 g MSO/kg, and primary skin irritancy was considered minimal. No skin sensitization was noted in guinea-pigs when MSO was applied to shaved skin under occlusion for 6 hr/day on nine consecutive days, followed by a challenge application 2 wk later. In the Draize test in the rabbit eye, conjunctival redness persisted between 1 min and 72 hr after instillation of 0·1 ml undiluted MSO into the conjunctival sac, but it had disappeared by day 7. No reaction was perceptible in the cornea or iris. Finally human patch testing was carried out in 50 subjects. Applications of MSO, each lasting for 24 hr, were made on the Monday, Wednesday and Thursday of three successive weeks, and then a challenge patch was applied 12 days later. No erythema, oedema or sensitization was detected.

A further investigation into the toxicity of MSO vapour (Parent, *ibid* 1979, **2,** 355) involved the exposure of groups of ten male and ten female rats to 0·15 or 0·45 g MSO vapour/m^3 generated at 180 or 225°C, respectively, for 6 hr/day, generally on 5 days/wk, over a 90-day period (actually 58 separate exposures). One male and one female in the lower-exposure group died after 7–9 wk, as did one of the males exposed to the higher MSO level. The cause of these deaths could not be determined because of autolysis, but they were not considered to be related to treatment. In females, but not in males, the growth rate was slightly impaired by exposure. No biochemical or haematological abnormalities attributable to exposure were detected. Organ weights expressed in relation to body weight did not differ significantly from control values in any of the treated groups, although the absolute weights of the spleen, kidney, lungs and pituitary were elevated in males exposed to 0·45 g MSO/m^3. There were no remarkable gross pathological findings, except minute red foci in the lungs of exposed animals. All the pulmonary lobes of one of the males that died during the study showed marked red discoloration, and blood was present in the thorax and abdomen of this animal. Histopathological findings were minimal in both test and control rats. Those in the lungs were generally indicative of respiratory infections and were less frequent in the MSO-treated groups than in the controls. On the other hand, focal lymphocytic infiltrates occurred more frequently, although they were no more severe in the livers of the treated rats than in those of the controls.

In order to assess the potential effect of long-term

contact with MSO during its use in photocopying, an 18-month skin-painting investigation was performed in mice (Parent, *ibid* 1979, **2,** 369). A dose of about 50 mg MSO was applied thrice weekly to the shaved skin of mice for 18 months without any effects on behaviour or weight gain or any signs of systemic toxicity becoming apparent. Lung adenomas and lymphosarcomas were found in both treated and control groups and showed no indication of any treatment-related effect. No skin papillomas were observed in the treated animals. The commonest non-neoplastic lesion encountered in both test and control animals was amyloidosis in various tissues. A significant increase in mortality was recorded in the treated females compared with the concurrent controls, although the difference was less marked in a comparison with historical controls. No such increase was seen in the treated males, but in both sexes a change in the slope of the control curves indicating a rise in mortality rate from about day 430 was accentuated in the curves for the MSO-treated groups. Whether this reflected some subtle effect of the test chemical remains questionable.

Taken as a whole, these studies do not suggest any marked toxicity on the part of this particular polydimethylsiloxane derivative. Since release agents used in the photocopying process may be carried out on the copy, skin contact seems likely to be the major route of occupational exposure to MSO, particularly in view of the material's low volatility. In this respect the results of the dermal toxicity, irritation and sensitization tests are encouraging. Without data on food consumption over the period following exposure, no conclusion can be drawn about the growth impairment shown by rats exposed once to an atmosphere containing 1·1 g MSO/m^3. There was little or no sign of this effect in other rats exposed repeatedly to lower concentrations (0·15 or 0·45 g m^3). The report that "no obvious effects on weight gain with dose level were noted" in the acute oral study is somewhat ambiguous. If some impairment in weight gain occurred in the orally treated animals that developed gastric and intestinal inflammation, it might be reasonable to suggest that some transient inflammation of the upper part of the gut could have resulted from the high-level inhalation exposure. This inflammation could have led to a depression of food intake sufficiently prolonged to affect the 14-day weight gain. Another point at issue in interpreting the inhalation studies is the identity of the material actually inhaled, since the low volatility of MSO necessitates the use of high temperature aeration, with the consequent possibility of thermal degradation. It appears, however, that MSO is no less stable than unsubstituted polydimethylsiloxanes and that the major volatiles produced from the two types of compound are the same (Parent, *Drug Chem. Toxicol.* 1979, **2,** 355), although it is possible that oxidative coupling of thiol groups to yield cross-linked higher molecular weight polymers of even lower volatility may also occur.

[P. Cooper—BIBRA]

THE 5-METHOXYPSORALEN DEBATE

For some time, bergamot oil, and more specifically, its 5-methoxypsoralen component has been associated with phototoxic effects on the human skin (Opdyke, *Fd Cosmet. Toxicol.* 1973, **11,** 1031). Subsequently caution was urged in the use of psoralens with ultraviolet (UV) light in the ('PUVA') treatment of psoriasis, on the grounds that somatic chromosomal damage might lead later to malignancy (Ashwood-Smith & Grant, *Br. med. J.* 1976, **1,** 342). This warning received support from a statement by Stern *et al.* (*New Engl. J. Med.* 1979, **300,** 809) that the risk of skin carcinoma was 2·63 times higher in psoriasis patients treated with oral 8-methoxypsoralen than that for a matched control group. The balancing of risks and benefits for psoriasis sufferers is fortunately not our concern here, and the question has already been considered in a more appropriate setting (Epstein, *ibid* 1979, **300,** 852). Our current interest lies in a related problem to which attention was drawn towards the end of 1979 and which has since given rise to spasmodic correspondence in the medical press.

Raising the question of the sale in some European countries of suntan preparations containing 5-methoxypsoralen, Ashwood-Smith (*Br. med. J.* 1979, **2,** 1144) suggested that the use of such formulations would probably lead to an increase in skin and other cancers. While admitting that there was no direct evidence that 5-methoxypsoralen, an effective tanning agent in the presence of light of 320–380 nm, had either carcinogenic or mutagenic properties, he claimed that there was nothing in its chemical structure to indicate that it would behave in a qualitatively different manner from other psoralens. The activities of psoralen itself or 8-methoxypsoralen were enumerated as the formation of monoadducts and cross-links with DNA in the presence of light, the production of point mutations and/or chromosomal mutations in a variety of cellular systems, and, in the presence of light, the production of skin cancer in mice and of skin and possibly other cancers in man.

This view was supported by Kersey (*ibid* 1980, **280,** 940) who maintained that while the use of psoralens might be acceptable in the treatment of psoriasis, they were unacceptable as tanning agents for fair-skinned individuals. Forlot (*ibid* 1980, **280,** 648), however, pointed out the value of pigmentation as a protection against carcinogenesis induced by UV burning and possibly against other types of damage and ageing of the skin. Referring to unpublished data accumulated by a manufacturer of a range of suntan preparations, he claimed that in several species the acute and subacute toxicity of 5-methoxypsoralen, with or without UV radiation, was much lower than that of 8-methoxypsoralen. Moreover, the intracellular distribution of the derivatives appeared to be different, the extent of reaction with nuclear material seemed to differ in quantity and perhaps also in the kind of binding, and

interim findings in a study of sunburn cells in hairless mice indicated that significantly more nuclear damage was caused by 8- than by 5-methoxypsoralen.

One response to Forlet (*loc. cit.*) pointed out that 5-methoxypsoralen is usually incorporated into suntan preparations in the form of natural bergamot oil rather than as the pure chemical (Grupper & Beretti, *ibid* 1980, **280,** 940) and stated that both the 8-methoxy and to a lesser extent the 5-methoxy derivative had given weakly positive results in the Ames test, with reactions comparable to those of caffeine. These correspondents cited three European studies, involving altogether over 8000 psoriasis patients, among whom no cases of cutaneous cancer attributable to PUVA therapy had been reported, in contrast to the findings of Stern *et al.* (*loc. cit.*). Moreover, an epidemiological study carried out in the bergamot-oil-producing area of Calabria had shown no increased incidence of cancer among subjects in contact with the oil. Further unpublished data, also cited by Grupper & Beretti (*loc. cit.*), concerned with interim findings in a study of hairless mice treated with a suspect suntan product and irradiated with a solar simulator. After 26 wk of treatment there was no indication of carcinogenicity.

As the pendulum seemed to be swinging in favour of 5-methoxypsoralen, Ashwood-Smith *et al.* (*Nature, Lond.* 1980, **285,** 407) presented the results of some comparative studies on 5- and 8-methoxypsoralens. When tested at a level (40 μg/ml) reported to be similar to those in suntan preparations, 5-methoxypsoralen was found to produce lethal and mutagenic (base-pair substitution) photosensitization of *Escherichia coli* in the presence of UV light and to induce frame-shift mutations in *E. coli* in the absence of light. At the same concentration it had both lethal photosensitizing effects and caused sister-chromatid exchanges in mammalian (Chinese hamster) cells in culture in the presence of UV light. The activity of 5-methoxypsoralan was generally less than that of the 8-methoxy derivative. In a note added in proof Ashwood-Smith *et al.* report that a study in France has shown that 5-methoxypsoralen in combination with near UV light is almost as potent a skin carcinogen in mice as 8-methoxypsoralen.

Another implication of the effects of methoxypsoralens on bacteria was raised by Hook (*Br. med. J.* 1980, **280,** 1537), who suggested that the genetic changes that methoxypsoralens may generate in skin organisms may be clinically important with regard, for example, to the acquisition and transfer of antibiotic resistance. Hook also reported that the psoralens problem may not be limited to tanning preparations, citing a study in which more than half of 108 perfumes were found to contain 5-methoxypsoralen at levels ranging from 0·00004 to 0·0108%.

It is unfortunate that much of the evidence that has been put forward hinges on the results of unpublished studies. Until these data are published and until the results of further animal studies become available the degree of hazard resulting from the use of suntan preparations containing 5-methoxypsoralen remains uncertain.

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ABSTRACTS AND COMMENTS

NATURAL PRODUCTS

Aflatoxin strikes the primate liver

Sieber, S. M., Correa, P., Daigard, D. W. & Adamson, R. H. (1979). Induction of osteogenic sarcomas and tumors of the hepatobiliary system in nonhuman primates with aflatoxin B_1. *Cancer Res.* **39,** 4545.

We have previously reported (*Cited in F.C.T.* 1979, **17,** 408) that in a long-term study on the effects of aflatoxin B_1 (AFB_1) three out of 42 monkeys treated with the aflatoxin for more than 2 yr developed primary liver tumours. This long-term study has been continued and an updated report is given in the paper cited above.

A total of 47 monkeys, mainly rhesus and cynomolgus, have now been treated for more than 2 months with AFB_1 at levels of 0·125–0·25 mg/kg intraperitoneally (ip) (once or twice weekly) and/or 0·1–0·8 mg/kg orally (once weekly, or 2–5 times/wk every 2 or 3 wk). Twelve are still alive and show no evidence of tumours. Of the 35 that have been autopsied so far, 13 (28% of the total population) developed a total of 17 malignant tumours. These included five primary liver tumours (two hepatocellular carcinomas and three haemangioendothelial sarcomas) and two bone sarcomas. [Included in these figures are the previously reported liver tumours observed in three of the animals (*ibid* 1979, **17,** 408).] There were also six carcinomas of the gall bladder or bile duct, three of the pancreas or pancreatic ducts, and one of the bladder. All of the tumour-bearing animals were either dosed orally or both orally and ip. The total dose of AFB_1 ranged from 99–1354 mg (mean 709 mg) given over 47–147 months (mean 114 months). Among 22 autopsied monkeys without tumours, 15 showed histological signs of liver damage including toxic hepatitis, cirrhosis, or hyperplastic liver nodules after total doses of AFB_1 ranging from 0·35–1368 mg (mean 363 mg) given over 2–141 months (mean 55 months).

These results indicate that AFB_1 is a potent hepatotoxin and carcinogen in nonhuman primates. It may also be concerned in the pathogenesis of tumours of the pancreas, gall bladder and bone.

AGRICULTURAL CHEMICALS

Chlorinated insecticides and the diet

Lakshmanan, F. L., Pommer, A. & Patterson, O. (1979). Chlorinated hydrocarbon insecticide residues in tissues of rats before and after reduction of body fat by dietary restriction. *J. agric. Fd Chem.* **27,** 720.

Starvation may lead to the mobilization of DDT from the fat depots of mammals and acutely toxic concentrations may be released into the bloodstream causing damage to the central nervous system. Dale *et al.* (*Toxic. appl. Pharmac.* 1962, **4,** 89) showed that in rats DDT can be mobilized by starvation, and in a later study it was found that similar effects occurred in cockerels (*Cited in F.C.T.* 1970, **8,** 442). In the study cited above the effects of various diets and of dietary restriction on the distribution of a mixture of chlorinated hydrocarbon insecticides (CHIs) was investigated.

Male rats were fed *ad lib.*, from weaning until 250 days of age, one of three nutritionally adequate diets with or without the addition of 2·8 ppm of a mixture of CHIs containing DDT, TDE, DDE, lindane, dieldrin and perthane. One of the diets (I) was a high-fat diet in which lard was the only nonprotein energy source. The other two diets (II and III) were high-sucrose, low-fat diets; the fat in II was mainly vegetable fat, whereas in III it was mainly animal fat. Seven or eight animals from each group were killed on day 250. From days 251 to 300 half of the remaining animals (12–15 in each group) were given only 50% of their previous food intake and after 300 days all of the animals were killed and the concentrations and total deposits of each compound in adipose tissue, liver and brain were determined by gas chromatography. (Perthane was not determined by the method used.)

Small amounts of all of the insecticides studied were detected in the control diets, but these levels were very low in comparison with those in rats fed CHI. Food intake, body-weight loss, organ weights, and carcass fat and water in animals given CHI were not significantly different from those of control animals. There were diet-associated differences in the amounts of CHI in the tissues. The rats consumed similar amounts of the two low-fat diets, but those given diet III deposited significantly more CHI, especially in the fat, than those on diet II. The food intake from diet I was lowest because of the high calorific content of this diet, but the amounts of CHI in the adipose tissue of animals given the high-fat diet were comparable to those in rats given diet II and the amount of CHI in the liver of rats given diet I was either comparable to or higher than the amount in the livers of rats given either of the low-fat diets. The amount of dieldrin in the brain of rats given diet III was significantly greater than that in rats given the other two diets, but diet had little effect on DDT, TDE and lindane contents of the brain.

Total DDT in adipose tissue, liver and brain was not altered by reduction of the dietary intake, regardless of which diet had been given. Concentrations of

DDE, TDE and dieldrin in the liver were generally unaffected by dietary restriction, since both total liver content of these insecticides and liver weight were decreased. With dietary restriction the amounts of DDE in the brain were significantly increased in rats given low-fat diets. The overall effect of diet, regardless of treatment, indicated that diet III resulted in the highest amount of dieldrin in adipose tissue, liver and brain. Thus, it seemed that the amount of fat in the diet, as well as body deposits of fat, may lead to differences in the levels of storage of CHI and CHI metabolites in adipose tissue, liver and brain.

Carcinogenicity of piperonyl butoxide still uncertain

Cardy, R. H., Renne, R. A., Warner, J. W. & Cypher, R. L. (1979). Carcinogenesis bioassay of technical-grade piperonyl butoxide in F344 rats. *J. natn. Cancer Inst.* **69,** 569.

Widespread human exposure to piperonyl butoxide (PB) arises from its use as a potentiator of pyrethrins and other insecticides. However, only equivocal results were obtained from a previous limited carcinogenicity study in which an increased incidence of lymph, liver and lung tumours was seen in one group of mice given technical-grade PB (cited by Haley, *Ecotoxic. envir. Safety* 1978, **2,** 9). In view of these facts, and because of its structural similarity to the known animal carcinogen, safrole, PB has won a well-earned place in the NCI's testing programme. The results of the rat study have now been published.

Groups of 50 male and 50 female Fischer 344 (F344) rats were given technical-grade PB in their diet at 5000 or 10,000 ppm for 2 yr. (The test material was 90% pure but safrole was not one of the 21 contaminants detected by gas chromatography.) The upper treatment level was chosen on the basis of a preliminary 8-wk feeding study to be the maximum tolerated dose. Whilst all groups had a high total incidence of tumours—for example all 20 of the control males had at least one tumour as did 15 out of 20 control females—PB did tend to reduce the time to first tumour in both sexes. A number of tumour types occurred in treated animals that were not found in controls and of these the following were singled out for comment by the investigators. Mesothelioma of the vaginal tunic was observed in the testes of four rats (two in each of the PB groups), peritoneal mesothelioma occurred in one male given 5000 ppm PB, and squamous cell carcinomas were observed in the preputial gland of one high-dose male and in the clitoral gland of one high-dose female.

Malignant lymphomas and leukaemias, grouped together as lymphoreticular neoplasms, were the only specific class of tumour found in statistically greater numbers in the rats given piperonyl butoxide. They occurred in 5% of the female controls, and in 14 and 30% of the low- and high-dose females, respectively. By the Cochran-Armitage test this trend was shown to be a statistically significant linear dose-response; no similar trend was seen in the male rats. The biological relevance of this finding is obscured by the fact that lymphoreticular tumours are frequently seen in control F344 rats. The incidence of leukaemia reported in the literature was said to vary from 21 to 35% in aged females. However the incidence of lymphoreticular neoplasms in untreated female F344 rats in other NCI bioassays that were conducted at the same laboratory over the same period was lower at 9·69%, and evaluation of data from a large pool of female control animals (of the same age and strain) derived from all NCI bioassay data available at the time gave an incidence of 8·86%.

[An experimental protocol involving only a small concurrent control group (and a control group of 40 would certainly qualify for such a criticism) reduces the clarity of the biological baseline and decreases further the already limited sensitivity of an animal bioassay in detecting a weak carcinogen. However, historical control data do have some part to play in the interpretation of carcinogenicity data, and the authors' conclusion that the study provides only equivocal support for PB's carcinogenicity is sound. Nevertheless, just as a statistically significant response is not necessarily conclusive evidence of biological activity, the presence of a rare tumour type in a treated group may be strong circumstantial evidence of carcinogenicity, even though the incidence may not be statistically significant. Therefore, it is unfortunate that no information was given in the paper on the occurrence of historical controls of those tumours that were seen in the PB-treated animals but were not present in the concurrent control group.]

Mutagenicity of fumigants

Teramoto, S., Saito, R., Aoyama, H. & Shirasu, Y. (1980). Dominant lethal mutation induced in male rats by 1,2-dibromo-3-chloropropane (DBCP). *Mutation Res.* **77,** 71.

The agricultural fumigants 1,2-dibromoethane (DBE) and 1,2-dibromo-3-chloropropane (DBCP) have both been shown to be carcinogenic in experimental animals (*Cited in F.C.T.* 1975, **13,** 144; *Federal Register* 1978, **43,** 52775). DBE has also been shown to have adverse effects on reproduction in several species (*Cited in F.C.T.* 1980, **18,** 747) and there is evidence for the interference of DBCP in human spermatogenesis (*ibid* 1980, **18,** 548). Both fumigants induce mutations in bacterial systems (*Federal Register* 1977, **42,** 63134; *cited in F.C.T.* 1980, **18,** 205; Rannug *et al. Chemico-Biol. Interactions* 1979, **24,** 265) and DBE has been shown to induce sex-linked recessive lethal mutations in *Drosophila melanogaster* males (Kale & Baum, *Mutation Res.* 1979, **68,** 59). In the study cited above the mutagenic potential of DBE and DBCP was investigated by dominant lethal assay in rats and mice.

Groups of 15 male rats were dosed with 10 or 30 mg DBE/kg, or 10 or 50 mg DBCP/kg by gastric intubation once daily for five successive days and were then mated overnight with an untreated virgin female, once a week, for 10 wk. Similar treatment of groups of 7–9 mice with 100 or 150 mg DBE/kg or 50–150 mg DBCP/kg was carried out daily for 5 days. Each male was then allowed to mate with two untreated virgin females for 7 days. Females were caged separately when a vaginal plug was observed

and at the end of each week any females remaining in the male's cage were removed and replaced by two new females. This mating procedure was continued for 6 wk. The pregnant females of both species were killed 12–14 days after conception and the numbers of corpora lutea, implants, live embryos and early and late embryonic deaths were scored, and for each treatment group the frequency of induced dominant lethal mutations was calculated weekly.

DBE and DBCP did not affect the mating performance of the male rat; the frequency of fertile matings was unaffected. DBE had no significant effect on the mean numbers of implants or live embryos and the incidence of dead implants was within normal limits. In the DBCP-treated group the mean numbers of corpora lutea and implants were comparable to those of the control group (intubated with olive oil). However, an increased incidence of dead implants (a criterion for induction of dominant lethals) with a corresponding decrease in the number of live embryos was observed in the females that were mated 1–6 wk after the males had been treated with 50 mg DBCP/kg with the highest incidence occurring among those mated 4–5 wk after treatment of the males. The estimated dominant lethal mutation index in this dose group was high for matings 4 and 5 wk after treatment but was low for matings 6 wk after treatment. At the lower dose level there was a significant increase in the frequency of dead implants from matings 4 and 5 wk after treatment. These results indicated that in rats DBCP exerted mutagenic activity in the post-meiotic stage of spermatogenesis, and particularly in the early spermatid stage.

In the mouse, neither DBE nor DBCP reduced the frequency of fertile matings nor did they have a significant effect on any of the parameters. Ethyl methanesulphonate which was given intraperitoneally as a positive control to 15 rats (200 mg/kg) and 7 mice (300 mg/kg) clearly induced dominant lethal mutations in both species.

The apparent lack of mutagenic activity of DBE, in the light of positive results using other test systems, was thought to reflect a difference in the sensitivity of the tests used, although the possibility of detoxication of the material before it reached the male germinal cells could not be discounted. The species difference in the activity of DBCP was believed to reflect the species-dependent metabolic pathways and the question of the potential of DBCP to cause genetic effects in man remains to be answered.

OCCUPATIONAL HEALTH

Chromium and the kidneys

Mutti, A., Cavatorta, A., Pedroni, C., Borghi, A., Giaroli, C. & Franchini, I. (1979). The role of chromium accumulation in the relationship between airborne and urinary chromium in welders. *Int. Archs occup. envir. Hlth* **43,** 123.

The nephrotoxic effects of occupational exposure to chromium have been studied by means of urinary indicators of renal-tubule lesions, such as β-glucuronidase, and of altered tubular metabolism, such as proteins, lysozyme and glucose (*Cited in F.C.T.* 1980, **18,** 102). Further studies of welders exposed to chromium fumes offer evidence that may be useful in assessing the degree of exposure of workers in industry.

The exposure to chromium of 22 welders working with high-chromium alloy electrodes was assessed and compared with urinary excretion of possible indicator compounds. Two subjects were monitored for urinary excretion of chromium for 1 month after their initial exposure to welding fumes. In the other 20 welders, urinary excretion of chromium was measured at various intervals after the end of exposure; the relationship between airborne and urinary concentrations of Cr was studied at different levels of chromium body burden; and proteins, β-glucuronidase and lysozyme in urine were measured in order to assess their value as early indicators of nephrotoxicity. The percentage of chromium in the welding fumes varied from 3–6%. Exposure was mainly to hexavalent chromium during welding with coated electrodes, whereas the percentage of the metal released in the hexavalent state during continuous-wire welding in a metal inert gas atmosphere was much lower. However, when workers used the latter method without fume removal systems (the use of which is said to decrease the quality of the welding) the net exposure to hexavalent chromium was similar to that of the group using coated electrodes.

There was a close correlation between airborne and urinary chromium concentrations and this suggested that urinary excretion above baseline values at the end of a period of exposure reliably indicates at least the rate of hexavalent chromium absorption. Urinary excretion of chromium increased with increasing body burden of chromium. No dose-response relationship could be detected between the level of chromium exposure in fumes and the degree of renal function impairment.

Lung cancer caused by chloromethyl ether

Weiss, W., Moser, R. L. & Auerbach, O. (1979). Lung cancer in chloromethyl ether workers. *Am. Rev. resp. Dis.* **120,** 1031.

Chloromethyl methyl ether (CMME) used as an alkylating agent in organic synthesis is weakly carcinogenic in animals. Industrial CMME is contaminated with bis-chloromethyl ether (BCME), a more powerful carcinogen, and so workers are usually exposed to both agents simultaneously. This has rendered the toxicology of the compounds somewhat uncertain (*Cited in F.C.T.* 1976, **14,** 650). Both CMME and BCME break down in the presence of water to form formaldehyde and hydrochloric acid. CMME yields methanol also. Some of the effects of CMME and BCME may be attributable to these breakdown

products (*ibid* 1977, **15**, 244). Further information about the types of tumour induced by exposure to CMME and BCME may elucidate the position.

Male workers in a chemical plant who had been exposed to both CMME and BCME (the mixture being designated chloromethyl ether, CME) between 1948 and 1960 were studied from 1960–1975. In 465 exposed workers the relative risk of lung cancer was 2·15 times that in unexposed controls and the general population and there was a dose-response relationship. However, the significant increase was limited to men with moderate or heavy CME exposure, the standardized mortality ratios for these two groups being 6·92 and 30·00 respectively. The higher cumulative chemical doses were largely the result of duration rather than of intensity of exposure. The incidence of lung cancers among men with a history of little or no exposure to CME seemed to be associated with smoking. On the other hand the cases of lung cancer among men moderately or heavily exposed to CME occurred in increasing proportions of men who had never smoked. Lung cancers in the general population and in chemical workers who had not been exposed to CME comprised some 45% squamous-cell carcinomas, 20% small-cell carcinomas, and 20% adenocarcinomas. The proportion of small-cell carcinomas in lightly exposed workers resembled that in controls and although there was a predominance of adenocarcinomas in this group its significance cannot be interpreted from the small number of cases involved. However, lung cancer in moderately and heavily exposed workers was almost always of the small-cell type. Induction of such tumours appears to be a specific response to significant exposure to CME.

Another threat to hairdressers

Borum, P., Holten, A. & Loekkegaard, N. (1979). Depression of nasal mucociliary transport by an aerosol hair-spray. *Scand. J. resp. Dis.* **60**, 253.

Exposure to hair sprays has been linked with lung diseases and there is evidence that polyvinylpyrrolidone (PVP) and related co-polymers may be the constituents responsible (*Cited in F.C.T.* 1980, **18**, 555). Another possible harmful effect of hair sprays, which may also be attributable to their polymer content, is described in the paper cited above.

In 15 healthy subjects (12 women and three men) aged 19–50 yr and without any detectable nasal abnormality, the rate of mucociliary transport in the nose was determined by measuring the time taken for saccharin placed on the inferior nasal concha to reach the taste receptors with the subject in the sitting position. A hair spray or a control Freon propellant aerosol was sprayed in the normal manner onto the hair for 20 sec while the subject inhaled normally every 5 secs. In 20 sec 15·6 g of hair spray or 15·7 g of Freon propellant were delivered. The spray (Resyn-28-1310), of which the constituents are not stated, reduced the mucociliary transport rate by some 30%. Significant reductions were found 15, 30 and 60 min after exposure, restoration to normal values taking 90 min. Freon alone produced no detectable effect at 15 and 30 min. Two subjects complained of headache and nausea after exposure to the hair spray but not after the Freon treatment.

The compound responsible for the effect has not been identified, but it is possible that the polymers (e.g. PVP, polyvinyl acetate) or the solvent (mainly ethanol) in the spray may affect the rheological properties of the mucus and thus mucociliary transport. A reduction in the rate of mucociliary transport decreases the effectiveness of the body's defence mechanisms against inhaled viruses, bacteria and carcinogenic compounds. Therefore it is a matter of concern that the results of this study suggest that it is possible that in professional hairdressers mucociliary transport may remain depressed throughout their working day, and possibly longer, and further studies would seem to be warranted.

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

The case against using surma

Aslam, M., Healy, M. A., Davis, S. S. & Ali, A. R. (1980). Surma and blood lead in children. *Lancet* **I**, 658.

Cosmetic preparations known as surma often contain lead sulphide, and increased blood lead levels have been reported in Asian children on whose eyes surma has been used (*Cited in F.C.T.* 1979, **17**, 310). The degree of hazard represented by the use of surma has been debated (Attenburrow *et al. Lancet* 1980, **I**, 323; Bakhshi, *Br. med. J.* 1978, **2**, 1159). However the authors cited above remain convinced that the use of surma does present a significant, practical risk to health.

The lead content of eight commercial surmas was determined, by atomic absorption spectrophotometry, to vary from <0·5% to >80% (mean 59·8). Analysis of a further 72 samples of home-made surmas obtained in Nottingham, Bradford, Manchester, Birmingham and London showed that 46 contained 20–86% lead (mean 54%). Transcorneal transport of lead is not possible, but passage of lead down the nasolachrymal duct and transfer *via* the fingers to the mouth are possible routes of absorption. At each application some 20 mg surma are deposited on the conjunctiva, and it is estimated that about 0·2% of the applied material is ingested. If surma containing 50% lead were applied once daily, the weekly dose of lead would be some 140 μg, and, assuming that 25–50% of ingested lead is absorbed in the gut, surma would contribute 37–70 μg lead/wk to the total body burden of lead in a child. The small size of the particles of most lead-containing surmas promotes their gastrointestinal absorption.

Among children in Nottingham, the 37 surma users had mean blood lead levels of 1·65 μmol/litre compared with 0·98 μmol/litre in the 25 controls (*Cited in*

F.C.T. 1979, **17**, 310). In Bradford, 14% of 117 Asian children (of whom 45 had used surma) had blood lead levels above 1·45 μmol/litre, and 5% had levels above 1·7 μmol/litre. In one child observed in hospital, and whose mother continued to apply surma despite medical advice not to do so, blood lead levels rose during 1 yr from 2·4 to 3·0 μmol/litre and in a second year to 3·5 μmol/litre.

ENVIRONMENTAL CONTAMINANTS

SCE in arsenic-exposed human lymphocytes

Zanzoni, F. & Jung, E. G. (1980). Arsenic elevates the sister chromatid exchange (SCE) rate in human lymphocytes in vitro. *Arch. dermatol. Forsch.* **267**, 91.

Arsenic exposure has been linked with the development of skin, lung and lymphatic cancers (*Federal Register* 1975, **40**, 3392), angiosarcoma of the liver (*Cited in F.C.T.* 1976, **14**, 507) and various internal malignant neoplasms (*ibid* 1979, **17**, 309). In addition, disturbances of nuclear division and somatic point mutations have been reported in lymphocyte cultures treated with low levels of inorganic arsenic (Petres & Berger, *Arch. dermatol. Forsch.* 1972, **242**, 343). Paton & Allison (*Mutation Res.* 1972, **16**, 332) also found that subtoxic doses of arsenic caused chromosome damage in human cells, and suggested that arsenic might have a dual effect by which it became incorporated into the DNA, so forming a weak link, and also inhibited repair enzymes.

Beckman *et al.* (*Envir. Hlth Perspect.* 1977, **19**, 145) found chromosomal aberrations in smelter workers exposed to arsenic (and other metals) and Burgdorf *et al.* (*Hum. Genet.* 1977, **36**, 69) reported an elevated rate of sister chromatid exchanges (SCEs) in the lymphocytes of patients treated with arsenic many years before. In the study cited above the effect of trivalent inorganic arsenic (Na_2HAsO_4) in SCE rates in cultured human peripheral blood lymphocytes from healthy individuals not previously exposed was investigated.

Preliminary screening tests were carried out on lymphocytes from three subjects in order to determine the optimum range of arsenic concentrations for SCE counting. The number and quality of observed metaphases increased with successive dilutions of the arsenic solution from 10^{-4} to 10^{-7} M. Whereas the addition of 10^{-4} M arsenic killed all of the lymphocytes, concentrations of 2×10^{-5}, 10^{-5}, 2×10^{-6} and 10^{-6} M induced chromosome aberrations including pulverization and breaks. SCEs were seen only in the arsenic-free controls and at arsenic dilutions of 10^{-7}, 10^{-6} and 2×10^{-6} M.

Cultures of lymphocytes from each of seven healthy volunteers were exposed to 0, 10^{-7}, 10^{-6} or 2×10^{-6} M arsenic. A dose-dependent increase in SCE per metaphase was observed and this increase was statistically significant at the 2×10^{-6} M dose level. The authors conclude that the evidence produced in previous work is confirmed and that these findings further implicate inorganic arsenic in the aetiology of multiple premalignant and malignant tumours observed in subjects exposed to arsenic.

Tissue reactions to PVC

Pigott, G. H. & Ishmael, J. (1979). A comparison between *in vitro* toxicity of PVC powders and their tissue reaction *in vivo*. *Ann. occup. Hyg.* **22**, 111.

One of two polyvinyl chloride (PVC) dust samples was strongly haemolytic *in vitro*, but since the activity was greatly reduced by washing it was attributed to some readily-soluble agent on the surface of the particles (*Cited in F.C.T.* 1976, **14**, 158). Neither of the samples was found to affect fibroblasts in culture. However, there have been isolated reports of pneumoconiosis in PVC workers (e.g. Arnaud *et al. Thorax* 1978, **33**, 19; Szende *et al. Medna Lav.* 1970, **61**, 433) and lung damage was found in rats and guinea-pigs after 2–7 months continuous exposure to PVC dust in a bagging plant (Frongia *et al. ibid* 1974, **65**, 321) at concentrations that have been calculated to be up to 30 times the ACGIH limit for a nuisance dust. In no case was it clear whether the lung changes were due to PVC *per se*, rather than to some other component of the dust, and further studies of the biological activity of various PVC dust samples have now been undertaken.

Powders from four PVC suspension polymers, three PVC emulsion polymers and one vinyl chloride–vinyl acetate suspension copolymer were suspended in physiological saline and ball-milled for 40 hr to reduce some of the particles to below 10 μm in diameter. Some samples of each polymer dust were allowed to stand overnight in saline and were then recovered by centrifugation, the residue was washed by centrifugation with saline and finally resuspended in fresh saline. Aliquots of the washed and unwashed suspensions were added to rat peritoneal macrophages *in vitro*, at a concentration of 0·5 mg dust/10^6 cells. After 2 hr, 0·5% trypan blue, which is excluded by living cells, was added to allow the proportion of dead dust-bearing cells to be estimated. Previous experience with a wide range of dusts had suggested that those causing less than 15% cell death *in vitro* were inert *in vivo*, and only two of the emulsion paste polymer samples exceeded this limit, causing cell mortalities of about 40 and 50% whether saline-washed or not. α-Quartz, which was used as a positive control, also caused 50% mortality.

Since washing with alcohol was subsequently found to reduce cell mortality from the two toxic samples to below the 15%, the responsible component was suspected to be the alcohol-extractable emulsifier, an alkylbenzene sulphonate. This was shown to cause complete cell death at 50 ppm, and 63% cell mortality even at a concentration of 5 ppm. Moreover, a non-

toxic PVC and a polymethylmethacrylate powder, stirred with a 10% (w/v) alcoholic solution of this emulsifier and then washed with saline to reduce contamination to below 0·1 ppm, still produced 17 and 28% cell mortalities, respectively.

Saline suspensions of two of the suspension polymers, one of the emulsion polymers that had produced cytotoxic effects *in vitro*, and the copolymer were injected into rats in single intraperitoneal (ip) or intratracheal doses of 20 or 2 mg respectively, and the rats were killed after 1, 3 or 6 months. Foci of granular refractive material surrounded by either a thin fibrous capsule or a mild foreign body reaction were found in the omentum and on the surfaces of the abdominal viscera and diaphragm of some of the ip-treated rats and in the alveoli and alveolar ducts in some of the intratracheally-treated rats. Small aggregates of inflammatory cells without obvious refractive material were also seen in the peritoneum of some ip-treated animals, but there were no reactions attributable to PVC in the lymph nodes, liver or spleen. There was no evidence of progressive fibrosis and there were no reactions attributable to PVC in the drainage lymph nodes in any of the treatment groups. In contrast, α-quartz, given by either route produced a progressive silicotic reaction. The PVC emulsion polymer sample that was cytotoxic *in vitro* gave reactions no different from the other three *in vivo*, suggesting that the *in vitro* result was a 'false positive' from the predictive point of view.

Carcinogenicity of dyestuff intermediates

Murthy, A. S. K., Russfield, A. B., Hagopian, M., Monson, R., Snell, J. & Weisburger, E. K. (1979). Carcinogenicity and nephrotoxicity of 2-amino-1-amino-2-methyl-, and 2-methyl-1-nitro-anthraquinone. *Toxicology Lett.* **4,** 71.

This paper reports the NCI's findings that the dyestuff intermediates 2-aminoanthraquinone (AA; *Federal Register* 1978, **43,** 51451), 1-amino-2-methylanthraquinone (AMA; *ibid* 1978, **43,** 47289) and 2-methyl-1-nitroanthraquinone (MNA; *ibid* 1978, **43,** 9359) were carcinogenic in mice and/or rats. All three compounds have previously been screened for mutagenic potential in *Salmonella typhimurium* but only MNA gave positive results (Brown & Brown, *Mutation Res.* 1976, **40,** 203). AA was not found to be carcinogenic in a limited study in which female rats were given the maximum tolerated dose in ten equal doses by gastric intubation and then observed for 9 months (Griswold *et al. Cancer Res.* 1968, **28,** 924). However, oral administration of AA has been associated with renal changes in female rats (Griswold *et al. loc. cit.*; Baker *et al. J. Toxicol. envir. Hlth* 1975, **1,** 1) and in the NCI study nephrotoxicity is reported to be induced by AA in female rats and by AMA in mice.

Groups of 50 male and 50 female Fischer 344 and $B6C3F_1$ mice were given one of the test compounds in their diet for 18 months at one of two dose levels, and then were given stock diet for 6 months (rats) or 4 months (mice). The high dose was equivalent to that which caused no deaths, no gross abnormalities and a 20% loss in body weight compared with the controls in preliminary 90-day studies. The doses for rats were as follows: 1 or 2% AA; 0·12 or 0·24% AMA; 0·06 or 0·12% MNA. The mice were given 0·5 or 1·0% AA, 0·03 or 0·06% AMA, or 0·03 or 0·06% MNA. However, since many of the female rats given AA died in less than 25 wk, two additional groups, given 0·2% AA (50 rats) and 0·5% AA (11 mice) were included. Rats (50 male and 50 female) and mice (50 male and 50 female) fed only the stock diet served as controls.

All three analogues of anthraquinone were carcinogenic to the rats but only AA and MNA produced neoplasms in mice. Hepatocellular neoplasms (neoplastic nodule and hepatocellular carcinoma) were common in all of the treated rats but they were most predominant in those fed AMA; of these, 36 out of 95 rats examined from the 0·12% dose group and 35 out of 92 rats from the 0·24% dose group bore this type of tumour. The incidence of hepatocellular neoplasm was slightly less in male rats treated with AA but only 1 out of 102 female rats treated with AA had a hepatocellular carcinoma. Hepatocellular neoplasms developed in mice treated with AA. Dose-related changes ranging from hyperplasia to adenosarcoma were found in the kidneys of rats treated with AMA whereas MNA was associated with an increased incidence of subcutaneous fibroma in rats and subcutaneous haemangiosarcoma in mice. Three of the male rats fed 0·12% MNA also developed haemangiosarcoma.

Renal lesions occurred predominantly in female rats fed AA and in mice of both sexes treated with AMA. Renal changes in the AA-treated rats were consistent with foreign body granulomatous nephritis (FBGN) and were accompanied by alopecia, mineralization of aorta and stomach and hypoplastic bone marrow. Nephrotoxicity in AMA-treated mice was manifested as glomerulonephritis and interstitial fibrosis. Renal adenosarcomas were found in association with these lesions in two male mice. Renal lesions were not found in rats or mice fed MNA.

The authors considered the stronger carcinogenic potential of AMA to be related to the position of a methyl group *ortho* to an amino group. They also noted that only the two analogues with an amino substitution in the one or two position were nephrotoxic.

[Details of statistical evaluations of the results are not given in this paper.]

Fd. Cosmet. Toxicol. Vol. 19, p. 279
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MEETING ANNOUNCEMENTS

MASS SPECTROMETRY SYMPOSIUM

The eighth international symposium on mass spectrometry in biochemistry, medicine and environmental research is to be held on 18–19 June 1981 in Venice, Italy. The meeting will cover the latest aspects of mass spectrometry and its areas of application. Further details may be obtained from A. Frigerio, Istituto de Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

COURSE ON TRACE-ORGANIC ANALYSIS

A workshop course and symposium entitled "Checking foodstuffs for trace organics" is to be held in Guildford, Surrey on 13–17 July 1981. The course which will include illustrative practical work will cover methods for the analysis of substances such as mycotoxins, nitrosamines, additives and packaging contaminants. The concluding symposium on 17 July will deal with novel or special approaches to such analytical work. Further details may be obtained from Dr E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH.

OCCUPATIONAL HAZARDS AND REPRODUCTION

An "International Course on Occupational Hazards and Reproduction" organized by the Institute of Occupational Health, Finland is to be held on 10–14 August 1981 in Espoo, Finland. The main topics to be covered are reproductive biology and toxicology, experimental reproductive studies, epidemiology particularly in relation to industrial hazards, and public policy with respect to reproductive hazards. The language of the course will be English although preference will be given to Nordic applicants. Further details may be obtained from Ms O. Teperi. Institute of Occupational Health, Haartmaninkatu 1, SF-00290, Helsinki 29, Finland.

Fd. Cosmet. Toxicol. Vol. 19, p. 280
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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:*

Enhancement of 1,2-dimethylhydrazine-induced colon carcinogenesis in mice by dietary agar. By H. P. Glauert, M. R. Bennink and C. H. Sander.

Subchronic effects of guar gum in rats. By S. L. Graham, A. Arnold, L. Kasza, G. E. Ruffin, R. C. Jackson, T. L. Watkins and C. H. Graham.

Testicular responses of rats and dogs to cyclohexylamine overdosage. By R. W. James, R. Heywood and D. Crook.

Effect of water-borne nitrate on salivary nitrite. By C. L. Walters and P. L. R. Smith.

Induction of tumours of the urinary system in F344 rats by dietary administration of sodium *o*-phenylphenate. By K. Hiraga and T. Fujii.

Toxicity of *Penicillium italicum* to laboratory animals. By N. P. J. Kriek and F. C. Wehner.

Lifespan oral toxicity study of vinyl chloride in rats. By V. J. Feron, C. F. M. Hendriksen, A. J. Speek, H. P. Til and B. J. Spit.

The presence of polychlorinated quaterphenyls in the tissues of Yusho victims. By T. Kashimoto, H. Miyata and N. Kunita.

Studies of the arylhydroxylation of monochlorophenylureas in the isolated perfused rat liver. By D. Westphal, K. Lucas and V. Hilbig.

A method for determining the maximum tolerated dose for *in vivo* cytogenetic analysis. By E. D. Thompson and R. A. Hiles.

Non-carcinogenic response to coumarin in Syrian golden hamsters. By I. Ueno and I. Hirono. (Short paper)

Diminution par un dithiocarbamate fongicide, le zinèbe, de l'activité des oxygénases microsomales du foie chez le rat: effets d'un régime à 9% de caseine. By M. A. Pélissier, F. Faudemay, E. Dooh-Priso, S. Attéba and R. Albrecht. (Short paper)

Multi-stage dose-response models in carcinogenesis. By F. W. Carlborg. (Review paper)

2-Acetylaminofluorene and the Weibull model. By F. W. Carlborg. (Review paper)

In utero exposure in chronic toxicity/carcinogenicity studies. By H. C. Grice, I. C. Munro, D. R. Krewski and H. Blumenthal. (Review paper)

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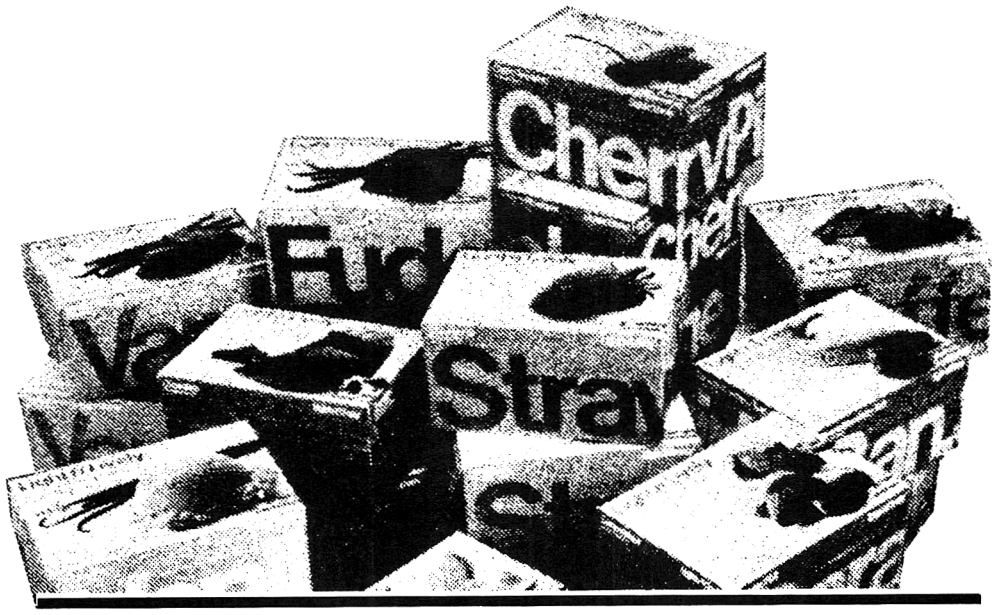

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