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# FOOD AND CHEMICAL TOXICOLOGY

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# *Research Section*

## SHORT-TERM TOXICITY STUDY OF METATARTARIC ACID IN RATS

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(*Received 10 July 1980*)

**Abstract**—Groups of rats were given metatartaric acid in the drinking-water in concentrations of 0 (control), 0·1, 0·5 or 3·0% for 18 wk. No effects associated with treatment were seen in the results of the haematological examinations and serum analyses. The treated animals consumed less water and food than the controls, probably because of the unpalatability of the test material. Administration of the 3% solution was associated in males with a reduced growth rate, some impairment of urine-concentrating ability during prolonged water deprivation (also seen in males on 0·5%) and histopathological changes in the stomach indicative of an inflammatory response in the submucosal layer. Both sexes of the 3% group showed an increase in relative kidney weight, without accompanying histopathological change. The no-untoward-effect level in this study was 0·1% metatartaric acid in the drinking-water, equivalent to a mean daily intake of 80 mg/kg body weight in the males and 130 mg/kg in the females.

### INTRODUCTION

Metatartaric acid, a product obtained by the fusion of tartaric acid at 170°C under reduced pressure, consists of mono and diesters of tartaric acid together with small amounts of free tartaric acids, pyruvic acid and unknown polyester acids, according to the Codex Oenologique International (1964). The material is used in wines to reduce the crystallization of calcium and potassium salts of tartaric acid.

In the UK the Food Additives and Contaminants Committee (1968) considered that although the product was likely to be transformed slowly into tartaric acid, some metatartaric acid would be present in wine as consumed. In the absence of adequate toxicological information, therefore, the Committee classified metatartaric acid as a Group E additive, i.e. an additive for which the available evidence was inadequate to enable an opinion to be expressed as to its suitability for use in food. However, subsequent consideration led to approval being given for the use of metatartaric acid at levels up to 100 mg/litre in wine in the UK (The Miscellaneous Additives in Food Regulations 1974, Statutory Instrument 1974, no. 1121). Use of the acid in wine was already permitted in parts of continental Europe, including France and Italy.

Our investigations into the stability of metatartaric acid showed that little hydrolysis to tartaric acid occurred in a 3% solution during an 8-hr period. In view of this observation, an 18-wk study was conducted on rats exposed to metatartaric acid administered in the drinking-water and the results are presented in this paper.

### EXPERIMENTAL

*Test material.* Metatartaric acid manufactured by Wormser Oenologie, France, was supplied by Newmark Chemical Laboratories, London. It complied with the requirements of The Miscellaneous Additives in Food Regulations 1974 (Statutory Instrument 1974, no. 1121) and had the following specification: an off-white powder; content, 105·4% tartaric acid equivalent; esterified tartaric acid, 27–35%; specific absorption $E^{1\%}_{1cm}$ 0·0076; specific rotation $[\alpha]_D^{20\,°C}$, +13·2; matter insoluble in water at 20°C, 1·48%; pyruvic acid, 53 ppm, arsenic, 0·02 ppm; lead, 0·16 ppm.

*Animals and diet.* Wistar rats obtained from a specified-pathogen-free breeding colony (SACI Laboratory Animal Division, Braintree, Essex) were fed ground Spratt's Laboratory Diet No. 1 *ad lib.* The animals were housed five per cage in air-conditioned rooms.

*Experimental design and conduct.* Groups of 15 male rats (body weight approximately 85 g) and 15 females (body weight approximately 75 g) were given *ad lib.* drinking-water containing metatartaric acid at levels of 0 (control), 0·1, 0·5 or 3·0% for 18 wk. Additionally, five rats of each sex were given 0, 0·5 or 3·0% metatartaric acid for 2 or 6 wk. The animals were weighed before the start of treatment, on days 1, 3 and 7 of the treatment period and subsequently at weekly intervals up to wk 18. Food and water intakes were measured over a 24-hr period preceding each weighing.

Urine was collected during the final week of treatment and examined for appearance, microscopic constituents and the presence of albumin, glucose, ketone bodies, bile salts and blood. A urinary concentration and dilution test was carried out at the same time by measuring the specific gravity and volume of urine voided during a 6-hr period of water deprivation and in a 2-hr period following a water load of 25 ml/kg body weight. Cells were counted in this urine specimen. In addition at wk 6 and 18 the same measurements were made on the urine collected during a 4-hr period commencing after water deprivation for 16 hr.

Table 1. *Mean body weights and overall food and water intakes for rats given 0–3·0% metatartaric acid in the drinking-water for 18 wk*

| Dose level (%) | Body weight (g) at day | | | | | | Mean food consumption (g/rat/day) | Mean water consumption (ml/rat/day) |
|---|---|---|---|---|---|---|---|---|
| | 0† | 28 | 56 | 87 | 105 | 126 | | |
| | | | | **Males** | | | | |
| 0 | 86 | 265 | 372 | 435 | 453 | 483 | 21·3 | 27·6 |
| 0·1 | 86 | 272 | 381 | 442 | 462 | 494 | 21·0 | 22·5** |
| 0·5 | 82 | 247* | 353 | 416 | 436 | 462 | 19·8** | 17·8** |
| 3·0 | 86 | 224*** | 316*** | 373*** | 392*** | 415*** | 17·1** | 15·0** |
| | | | | **Females** | | | | |
| 0 | 73 | 168 | 209 | 234 | 245 | 256 | 16·2 | 28·1 |
| 0·1 | 75 | 162 | 204 | 231 | 241 | 254 | 15·7 | 23·4** |
| 0·5 | 74 | 161 | 200 | 227 | 237 | 248 | 15·0* | 18·1** |
| 3·0 | 76 | 157* | 196 | 223 | 233 | 241 | 14·7* | 14·1** |

†Values on first day of dosing.

Body weights are the means for groups of 15 animals. Values for overall food and water consumption are derived from measurements (on three cages of five animals) made in the 24 hr preceding each weighing. Figures marked with asterisks differ significantly from the appropriate control value (Student's $t$ test for body-weight values and the ranking test of Wilcoxon & Wilcox (1964) for the mean food and water intakes): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

At the end of treatment the animals were killed by exsanguination from the aorta under barbiturate anaesthesia, following an overnight period without food. Samples of blood were obtained from haematological examination and serum analyses. The animals were examined for macroscopic abnormalities and the brain, pituitary, thyroid, heart, liver, spleen, kidneys, adrenal glands, stomach, small intestine and caecum (with and without its contents) were weighed. Samples of these organs and of oesophagus, trachea, lung, salivary gland, aorta, thymus, various lymph nodes, urinary bladder, colon, rectum, pancreas, prostate, seminal vesicles, uterus, spinal cord, skeletal muscle, sciatic nerve, eye and Harderian gland and other tissues that appeared abnormal were preserved in 16% buffered formalin. Paraffin-wax sections 5 $\mu$m thick were stained with haematoxylin and eosin. Microscopic examination of the sections was confined to tissues taken at 18 wk from all of the rats given 3% metatartaric acid and half of the animals in the 0·5% and control groups.

Blood samples were examined for haemoglobin content, packed cell volume and counts of erythrocytes and total and differential leucocytes. In the absence of treatment-related changes in the erythrocyte counts, reticulocytes were not examined. Differential leucocyte counts were carried out on all the groups at wk 2 but were confined to the control and highest treatment groups at wk 6 and 18. Serum was analysed for the content of urea, glucose, total protein and albumin and for the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases and lactate dehydrogenase.

## RESULTS

One female rat in the group given 3% metatartaric acid for 18 wk died during wk 2 and was replaced. No other deaths occurred during the treatment period and the animals appeared normal.

Reductions in the rate of body-weight gain were statistically significant in male rats given 3% metatartaric acid but not in other groups (Table 1). Throughout the experiment the intakes of food and water were lower in the treated groups than in the controls; the consequent reductions in the mean values for the whole period (Table 1) were dose related, those for water intake being statistically significant at all dose levels and those for food intake at the two higher doses. The mean intakes of metatartaric acid from the 0·1, 0·5 and 3% solutions were 0·08, 0·33 and 1·81 g/kg/day, respectively, for the male rats and 0·13, 0·52 and 2·52 g/kg/day for the females.

The haematological findings (Table 2) did not show any treatment-related effects. The observed reduction from control values in the erythrocyte count at wk 18 in male rats on 0·5% metatartaric acid was not accompanied by a corresponding change in the packed cell volume. Similarly, the reduced packed cell volume in the females given 3% was not associated with a comparable change in the red cell count. The results of the serum analyses were similar in the treatment and control groups.

The results of the urine analyses are shown in Table 3. Compared with the controls, rats given 0·5 or 3% metatartaric acid for 2, 6 or 18 wk excreted less urine of higher specific gravity when deprived of water for 6 hr or during the 2-hr period following a water load. In contrast, at wk 18, male rats given 0·5 or 3% metatartaric acid excreted urine of lower specific gravity in larger volumes than control animals in the 16–20 hr after the water load. No comparable effects were seen in the females. Cell excretion and urinary constituents were normal in the treated animals.

At autopsy, occasional lung lesions of the type seen in chronic lung disease were seen. The gastric mucosa was reddened in two male rats treated with the 3% metatartaric acid for 18 wk. No other abnormalities were found.

Some statistically significant differences from control values were observed in the recorded and/or relative weights of organs of rats given 3% metatartaric acid. The mean weights of the brain, liver, spleen, stomach, small intestine, pituitary and thyroid were reduced at one or more of the termination times, most commonly in the males at wk 2 and 18. However, in the calculation of relative weights, the low terminal

Table 2. *Mean haematological values in rats given 0–3·0% metatartaric acid in the drinking-water for 18 wk*

| Dose level (%) | Hb (g/100 ml) | PCV (%) | RBC ($10^6$/mm$^3$) | Leucocytes: Total ($10^3$/mm$^3$) | Leucocytes: Differential (%) N | E | L | M |
|---|---|---|---|---|---|---|---|---|
| **Males** | | | | | | | | |
| 0 | 16·5 | 47 | 8·2 | 4·7 | 32 | 1 | 66 | 1 |
| 0·1 | 16·6 | 47 | 7·9 | 5·1 | — | — | — | — |
| 0·5 | 16·5 | 46 | 7·5* | 4·7 | — | — | — | — |
| 3·0 | 16·4 | 46 | 7·7 | 5·0 | 28 | 1 | 70 | 1 |
| **Females** | | | | | | | | |
| 0 | 16·6 | 47 | 6·9 | 3·8 | 33 | 1 | 64 | 2 |
| 0·1 | 16·7 | 47 | 7·3 | 3·8 | — | — | — | — |
| 0·5 | 16·2 | 46 | 7·1 | 3·8 | — | — | — | — |
| 3·0 | 16·1 | 45** | 7·1 | 3·9 | 31 | 1 | 67 | 1 |

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
N = Neutrophils E = Eosinophils L = Lymphocytes
M = Monocytes

Figures are means for groups of 15 rats and those marked with asterisks differ significantly (Student's *t* test) from the control value: $*P < 0.05$: $**P < 0.01$. No effects were observed in the same series of examinations at wk 2 and 6.

Basophils did not account for more than 0·5% of the leucocytes in any group and inclusions were not seen in the erythrocytes.

Table 3. *Mean results of renal concentration/dilution tests and urinary cell excretion in rats given 0–3·0% metatartaric acid in the drinking-water for 2, 6 and 18 wk*

| Dose level (%) | Cell excretion ($10^3$/hr) | Concentration test: Specific gravity 0–6 hr | Specific gravity 16–20 hr | Volume (ml) 0–6 hr | Volume (ml) 16–20 hr | Dilution test (2 hr): Specific gravity | Volume (ml) |
|---|---|---|---|---|---|---|---|
| **Wk 2** | | | | | | | |
| Male | | | | | | | |
| 0 | 1·2 | 1·035 | — | 1·9 | — | 1·008 | 2·7 |
| 0·5 | 0·9 | 1·059** | — | 0·7* | — | 1·024 | 0·7 |
| 3·0 | 0·2 | 1·048 | — | 0·9 | — | 1·054 | 0·3 |
| Female | | | | | | | |
| 0 | 0·1 | 1·020 | — | 1·8 | — | 1·010 | 1·9 |
| 0·5 | 0·4 | 1·035 | — | 0·9 | — | 1·019 | 1·6 |
| 3·0 | 0·1 | 1·052 | — | 0·3* | — | 1·040* | 0·6 |
| **Wk 6** | | | | | | | |
| Male | | | | | | | |
| 0 | 0·2 | 1·023 | 1·072 | 3·4 | 0·6 | 1·007 | 5·7 |
| 0·5 | 0·3 | 1·043 | 1·081 | 1·8 | 0·4 | 1·018 | 4·0 |
| 3·0 | 0·1 | 1·071** | 1·077 | 1·2* | 0·9 | 1·023* | 2·1* |
| Female | | | | | | | |
| 0 | 0·3 | 1·022 | 1·055 | 2·1 | 0·6 | 1·009 | 3·7 |
| 0·5 | 0·1 | 1·051 | 1·077 | 0·4** | 0·3 | 1·024* | 2·0 |
| 3·0 | 1·9 | 1·064** | 1·080* | 0·6** | 0·4 | 1·019* | 1·2** |
| **Wk 18** | | | | | | | |
| Male | | | | | | | |
| 0 | 2·3 | 1·028 | 1·078 | 3·2 | 0·6 | 1·005 | 7·4 |
| 0·1 | 1·4 | 1·034 | 1·079 | 3·3 | 0·6 | 1·005 | 8·5 |
| 0·5 | 2·9 | 1·038 | 1·074 | 1·7* | 1·2*** | 1·004 | 6·9 |
| 3·0 | 1·9 | 1·062*** | 1·071** | 1·0*** | 1·0* | 1·018*** | 1·6*** |
| Female | | | | | | | |
| 0 | 1·3 | 1·039 | 1·077 | 2·0 | 0·7 | 1·006 | 5·1 |
| 0·1 | 1·1 | 1·025 | 1·073 | 1·9 | 0·4 | 1·007 | 3·8 |
| 0·5 | 2·4 | 1·034 | 1·076 | 2·2 | 0·5 | 1·005 | 4·7 |
| 3·0 | 0·9 | 1·057* | 1·075 | 0·7* | 0·6 | 1·019*** | 1·9*** |

Results are means for groups of five rats at wk 2 and 6 and groups of 15 at wk 18. Values marked with asterisks differ significantly (White, 1952) from those of the controls: $*P < 0.05$: $**P < 0.01$: $***P < 0.001$.

Tests for glucose, bile salts, blood and ketones were negative and the amount of protein was similar in the urine from the treated and control rats.

Table 4. *Relative organ weights of rats given 0–3·0% metatartaric acid in the drinking-water for 2, 6 and 18 wk*

| Dose level (%) | Relative organ weights (g/100 g body weight) | | | | | | | | | | | | | Terminal body weight (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Brain | Heart | Liver | Spleen | Kidneys | Stomach | Small intestine | Caecum Full | Caecum Empty | Adrenal† | Gonads‡ | Pituitary† | Thyroid† | |
| **Wk 2** | | | | | | | | | | | | | | |
| Male | | | | | | | | | | | | | | |
| 0 | 1·08 | 0·38 | 3·57 | 0·46 | 0·88 | 0·66 | 3·25 | 1·54 | 0·45 | 21·5 | 1·10 | 3·4 | 5·8 | 168 |
| 0·5 | 1·19** | 0·43 | 3·41 | 0·41 | 0·92 | 0·69 | 3·98 | 1·63 | 0·41 | 20·1 | 0·94 | 3·2 | 5·6 | 151 |
| 3·0 | 1·24** | 0·46** | 3·55 | 0·37 | 0·92 | 0·69 | 4·04 | 2·17 | 0·51 | 21·3 | 1·14 | 3·6 | 5·0 | 141** |
| Female | | | | | | | | | | | | | | |
| 0 | 1·25 | 0·44 | 3·61 | 0·33 | 0·86 | 0·71 | 4·35 | 1·76 | 0·45 | 28·1 | 46 | 4·4 | 6·7 | 129 |
| 0·5 | 1·34 | 0·47 | 3·51 | 0·33 | 0·94 | 0·66 | 3·94 | 1·59 | 0·52 | 27·2 | 48 | 4·4 | 6·0 | 124 |
| 3·0 | 1·28 | 0·42 | 3·80 | 0·33 | 0·95 | 0·75 | 3·86 | 1·89 | 0·54 | 30·6 | 34 | 4·8 | 6·3 | 122 |
| **Wk 6** | | | | | | | | | | | | | | |
| Male | | | | | | | | | | | | | | |
| 0 | 0·63 | 0·32 | 2·89 | 0·22 | 0·68 | 0·43 | 2·14 | 1·17 | 0·32 | 15·0 | 1·04 | 2·4 | 5·0 | 307 |
| 0·5 | 0·65 | 0·30 | 2·85 | 0·21 | 0·69 | 0·43 | 2·09 | 1·35 | 0·35 | 14·3 | 1·07 | 2·3 | 5·6 | 296 |
| 3·0 | 0·66 | 0·33 | 2·87 | 0·22 | 0·74 | 0·45 | 2·25 | 1·61* | 0·30 | 16·4 | 1·06 | 2·1 | 5·2 | 275 |
| Female | | | | | | | | | | | | | | |
| 0 | 0·92 | 0·37 | 2·84 | 0·27 | 0·74 | 0·58 | 2·83 | 0·96 | 0·35 | 28·4 | 38 | 5·8 | 5·8 | 197 |
| 0·5 | 0·94 | 0·36 | 2·90 | 0·26 | 0·78 | 0·52 | 2·70 | 1·41* | 0·37 | 27·8 | 44 | 4·5 | 4·9 | 195 |
| 3·0 | 0·93 | 0·38 | 2·99 | 0·27 | 0·80* | 0·55 | 3·49 | 1·55* | 0·51*** | 26·6 | 45 | 5·2 | 6·2 | 188 |
| **Wk 18** | | | | | | | | | | | | | | |
| Male | | | | | | | | | | | | | | |
| 0 | 0·47 | 0·23 | 2·20 | 0·17 | 0·51 | 0·38 | 1·73 | 0·77 | 0·25 | 10·1 | 0·77 | 2·1 | 3·5 | 460 |
| 0·1 | 0·46 | 0·23 | 2·21 | 0·17 | 0·54 | 0·38 | 1·67 | 0·82 | 0·26 | 9·3 | 0·73 | 1·9 | 3·1 | 471 |
| 0·5 | 0·46 | 0·23 | 2·11 | 0·17 | 0·53 | 0·38 | 1·76 | 0·77 | 0·25 | 9·8 | 0·79 | 2·1 | 3·7 | 445 |
| 3·0 | 0·54* | 0·26* | 2·26 | 0·22* | 0·62** | 0·43* | 1·84 | 1·04** | 0·34** | 11·8 | 0·92*** | 1·9 | 3·5 | 390*** |
| Female | | | | | | | | | | | | | | |
| 0 | 0·80 | 0·32 | 2·51 | 0·27 | 0·62 | 0·55 | 2·59 | 1·34 | 0·38 | 19·9 | 37 | 4·6 | 5·8 | 239 |
| 0·1 | 0·82 | 0·30 | 2·54 | 0·25 | 0·61 | 0·53 | 2·42 | 1·10 | 0·35 | 21·0 | 35 | 4·5 | 5·4 | 236 |
| 0·5 | 0·83 | 0·33 | 2·50 | 0·25 | 0·65 | 0·54 | 2·47 | 1·29 | 0·40 | 21·1 | 37 | 5·2 | 5·9 | 229 |
| 3·0 | 0·83 | 0·31 | 2·54 | 0·24 | 0·71*** | 0·53 | 2·41 | 1·45 | 0·42 | 22·5 | 40 | 4·8 | 5·8 | 226 |

†Weights of this organ are expressed in mg/100 g body weight.
‡Weights of female gonads are expressed in mg/100 g body weight.
Values are means for groups of five rats at wk 2 and 6 and of 15 rats at wk 18; those marked with asterisks differ significantly (Student's *t* test) from the control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

body weights of these animals more than offset these organ-weight reductions and on occasions the brain, heart, spleen, stomach and testes showed a statistically significant increase in relative weight (Table 4). Statistically significant differences in kidney weights were restricted to animals given 3% metatartaric acid. The recorded weights showed a lower value in males at wk 2 and an increase ($P < 0.05$) in females at wk 18, while the relative weights showed increases in females at wk 6 and 18 and in males at wk 18. Similar findings were recorded for the relative weight of the empty caecum, with statistically significant increases in females at wk 6 and in males at wk 18.

Histological examination revealed slight fatty change and small foci of necrosis in the liver of some of the treated animals and in controls. Rats of both sexes showed interstitial pneumonitis and minor renal tubular damage and the incidence and severity of these findings were similar in the treated and control rats. In the group of male rats given 3% metatartaric acid for 18 wk, polymorphonuclear infiltration of the submucosal layer of the stomach was observed in three animals and in two cases this was accompanied by hyperplasia of the non-glandular epithelium.

## DISCUSSION

The acidity of the compound in solution was sufficiently corrosive at 3% concentration to produce gastric irritation and probably rendered metatartaric acid solutions unpalatable, a likely explanation for the observed dose-related decrease in water consumption. The association between restricted water intake and reduced food consumption is well established (Cizek & Nocenti, 1965; Strominger, 1947) and may well explain the reduced food consumption recorded in animals drinking 0·5 or 3% metatartaric acid. The consequent depression in body weight was statistically significant in male rats at the 3% dose level throughout the study.

The major findings in this study were related to the kidneys. The increased weights of this organ cannot be confidently interpreted as an adverse effect in the absence of any treatment-related pathological lesions, and most of the changes noted in the urine tests can be explained in terms of the reduced water intake. However the apparent inability of males given 0·5 or 3% metatartaric acid to concentrate urine under conditions of prolonged dehydration may suggest some impairment of renal function. Even this may be related to the reduction in food intake, since this situation has been shown to affect renal function adversely in the rat without causing a detectable alteration in kidney histology (Sharratt & Frazer, 1963). In view of their equivocal nature, these findings are difficult to interpret, but the possibility of treatment-related adverse effects on the kidney cannot be excluded.

Most of the other organ-weight changes were encountered in animals with low body weights and are typical of those previously found in animals with low body weights due to a reduced intake of nutrients (Feron, de Groot, Spanjers & Til, 1973; Gray, Butterworth, Gaunt *et al.* 1977; Oishi, Oishi & Hiraga, 1979; Peters & Boyd, 1966). The increase in relative caecum weight at wk 6 in females and wk 18 in males on 3% metatartaric acid is of doubtful toxicological significance and may have resulted in part from the osmotic effect of the solution administered.

No treatment-related adverse effects could be deduced from the haematological findings. No treated group had both a low red cell count and a reduced packed cell volume.

In this study administration of a 3% metatartaric acid solution produced untoward effects in male and female rats in the form of irritation of the gastric mucosa, reduced body-weight gain and an increase in relative kidney weight. Additionally, the results of the urine concentration/dilution tests suggest a possible adverse effect on renal function at the 0·5 and 3% levels of administration. Thus, the no-untoward-effect level for metatartaric acid in this study was 0·1% in the drinking-water, providing mean intakes of 80 and 130 mg/kg/day for male and female rats, respectively.

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# MUTAGENICITY OF THE PRODUCTS OBTAINED FROM HEATED MILK SYSTEMS

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**Abstract**—Methylene chloride extracts of the browning reaction products prepared from model systems consisting of major milk components (casein and/or lactose, and non-fat dried milk) were tested for mutagenicity in the Ames Salmonella/microsome assay. Samples obtained by heating aqueous solutions of these components under either neutral or basic (pH 10) conditions exhibited no significant mutagenic activity when tested with or without S-9 mix. The addition of common food additives, such as sodium nitrite, butylated hydroxyanisole and butylated hydroxytoluene, to the aqueous solutions did not enhance the mutagenic activity of the browning samples. On the other hand, the tar samples prepared by heating the same milk components in the dry state exhibited strong mutagenicity, primarily to *Salmonella typhimurium* strain TA98 and only with S-9 mix. A casein/lactose mixture and non-fat dried milk were also heated with baking soda in the dry state. The presence of the baking soda enhanced the mutagencity of the browning products; the tar from the non-fat dried milk heated with baking soda was the most potently mutagenic of all the samples towards strain TA98 and also produced a positive response in strain TA100 in the presence of S-9 mix.

## INTRODUCTION

Non-enzymatic browning is the name given to the formation of brown-coloured products as a result of the heating or storage of protein with sugar. The typical reaction mechanisms occurring in the browning process have been investigated since the beginning of the century, when Maillard (1916 & 1917) recognized the brown chromophores produced from heated sugar–amine systems. These reactions have since been reviewed in depth by Hodge (1953). Reports of most studies concerned with non-enzymatic browning (Rizzi, 1972; Sakaguchi & Shibamoto, 1978; Shibamoto & Bernhard, 1978) have described the products, in terms of their characteristic flavours, which are considered desirable in many cooked foods. The food industry has undertaken such investigations as an aid in the development of more palatable food items for consumers.

Other studies have described browning products as compounds eliciting mutagenic responses in the Ames Salmonella/microsome assay, a short-term bacterial mutagenicity assay which has received much attention in the past decade. Spingarn & Garvie (1979) demonstrated the mutagenicity of several sugar–ammonia model systems (glucose, rhamnose, galactose, xylose, arabinose, 2-deoxyglucose) using the Ames *S. typhimurium* strain TA98 with a liver microsomal fraction (S-9) incorporated into the agar. A model system containing glucose and cysteamine produced browning products of significant mutagenicity towards *S. typhimurium* strains TA98 and TA100 with and without S-9 mix (Mihara & Shibamoto, 1980). Toda, Sakizawa & Shibamoto (1981) demonstrated the strong mutagenic activity of products from a rhamnose–ammonia–hydrogen sulphide model system towards strain TA98 with S-9 mix. A model system containing maltol and ammonia produced compounds procuring a mutagenic response from, again, strain TA98 with S-9 mix.

The formation of mutagenic materials in cooked foods has been reported by many researchers. Nagao, Honda, Seino *et al.* (1977a) reported the formation of mutagenic pyrolytic products in broiled foods, observing that tars obtained from charred fish and steak were more mutagenic than benzo[*a*]pyrene. Nagao, Yohagi, Kawauch *et al.* (1977b) reported the mutagenicity of tars obtained from various foods (egg-white protein, serum albumin, calf-thymus histone protein, calf-thymus DNA, potato starch and vegetable oil). Tars obtained from the pyrolysis of amino acids (including DL-tryptophan, L-serine and L-glutamic acid) exhibited strong mutagenicity (Nagao *et al.* 1977b).

The formation of tars in cooked foods is initiated by browning reactions (Hodge, 1953). It is thus possible that some browning products play an important role in the mutagenicity of tars from cooked foods. The structure of the mutagenic chemicals isolated from these tars is apparently related to browning products (Sugimura, 1980). Recently, Wei, Kitamura & Shibamoto (1981) reported that strongly mutagenic tars had been obtained from the starch/glycine browning system.

The model systems investigated previously were simple sugar–amine systems. The study described here involved a more complex, yet common, system which included the milk protein, casein, and the milk sugar, lactose. The dairy industry has devoted much time to studying non-enzymatic browning of milk and milk products in order to minimize browning during pasteurization, sterilization and dehydration processes. But whether or not milk and milk products undergo

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*Abbreviations*: BHA = Butylated hydroxyanisole; BHT = butylated hydroxytoluene; DMSO = dimethylsulphoxide.

browning processes prior to sale, they are commonly used by consumers in heated foods such as casseroles, cakes, omelettes and breads. Consequently, non-enzymatic browning of casein–glucose and casein–galactose model systems has been well characterized (Ferretti, Flanagan & Ruth, 1970; Lea & Hannan, 1949; Rao & Rao, 1972).

Because of past research implicating browning products as potential mutagens and the common use of milk and milk products in baking and frying, it was considered of interest to investigate the possible mutagenicity of browning products extracted from model milk systems.

## EXPERIMENTAL

*Reaction of casein and lactose in neutral aqueous solution.* An aqueous solution (250 ml) containing 17 g casein (from Sigma Chemical Co., St. Louis, MO) was adjusted to pH 7 with 0·1 N-NaOH solution prior to the addition of 24 g lactose (Sigma Chemical Co.), providing a ratio of these two components similar to that found in cows' milk (Johnson, 1978). The solution was then refluxed at 100°C for 2 or 6 hr. After cooling, the pH was lowered to 4 with acetic acid to precipitate unreacted casein, centrifuged (7000 **g**) at 10°C for 15 min and filtered in a Buchner funnel to remove unreacted casein and solid materials. The filtrate was readjusted to pH 7 with 0·1 N-NaOH and extracted with methylene chloride using a Soxhlet liquid–liquid continuous extractor for 20 hr. The methylene chloride fraction was dried over anhydrous sodium sulphate for 12 hr and filtered. The solvent was removed in a rotary flash evaporator under vacuum, and then in a stream of purified nitrogen. The tar was stored in a teflon-sealed vial in a freezer (−5°C) until assayed for mutagenicity.

For studying the effects of additional reactants on mutagenic activity, neutral aqueous solutions of casein and lactose prepared as described above were refluxed in the presence of $NaNO_2$ (0·3 mM) and/or BHA (1·75 mg) and BHT (1·75 mg).

*Reaction of casein and lactose in basic aqueous solution.* An aqueous solution (500 ml) containing 100 g casein and 100 g lactose was adjusted to pH 10 with 1 N-NaOH solution, to accelerate the browning reaction and was then heated for 6 hr under reflux. After cooling, the reaction solution without further pH adjustment was extracted with methylene chloride and stored, as described above.

*Dry-state reactions of casein and/or lactose or of non-fat dried milk.* Six separate experiments were carried out using the following systems: (I) 25 g casein; (II) 25 g lactose; (III) 12·5 g casein/12·5 g lactose; (IV) 10 g casein/10 g lactose/5 g baking soda; (V) 20 g commercial non-fat dried milk; (VI) 15 g commercial non-fat dried milk/5 g baking soda. For each experiment the reactants were placed in a 125 ml Erlenmeyer flask, thoroughly mixed and covered with glass wool. The top of the flask was attached to a glass-tube trap (15 cm × 1·5 cm ID), packed with 50/80 mesh porous polymer (Porapak Q; Waters Associates, Milford, MA) previously conditioned at 150°C for 1 hr. The flask was directly heated by flame using a Fischer burner; the temperature was 290°C at the bottom of the flask and 80°C at the reactant-glass wool interface. Purified nitrogen gas was introduced into the flask during the reaction ($N_2$ flow rate: 30 ml/min). The sample was heated until the entire reaction mixture was browned (approximately 30 min), and the reaction mixture and apparatus were then allowed to cool for 30 min. The Porapak Q trap tube was removed from the flask and flushed with nitrogen gas for an additional 30 min in order to remove all remaining water.

The tar sample in each flask (samples Ia–VIa) was then processed; the reaction flask with its contents was thoroughly crushed and this solid material was extracted with methylene chloride, using a Soxhlet extractor, for 20 hr. The samples (Ib–VIb) from the Porapak Q traps were prepared by subjecting the Porapak Q removed from the trapping tube to extraction with methylene chloride in a Soxhlet extractor for 20 hr. Each methylene chloride extract was dried over anhydrous sodium sulphate for 12 hr and filtered. The solvent was then removed in a rotary flash evaporator under vacuum. The resultant oily material was further concentrated in a stream of purified nitrogen. Each sample was stored in a teflon-sealed vial in a freezer (−5°C) until assayed for mutagenicity.

*Mutation tests.* The methods described by Ames, McCann & Yamasaki (1975) were used for the examination of each sample in a dose-response assay. *S. typhimurium* tester strains TA98 and TA100 were kindly provided by Professor Bruce N. Ames (Biochemistry Department, University of California, Berkeley, CA). Spot tests were used for examination of the sample obtained from the heating of mixtures in neutral aqueous solutions, 0·5 mg of sample in 20 μl DMSO being applied to a filter paper disc placed on top of agar containing the bacteria with or without S-9 mix immediately after agar solidification. The liver-enzyme S-9 mix was prepared by the method described by Ames *et al.* (1975). Plate incorporation assays were used to demonstrate dose-related responses. For these, 2 ml molten top agar containing 100 μl of an overnight culture of *S. typhimurium* strain TA98 or TA100, 500 μl cofactor solution with or without 10% S-9 mix and 20 μl DMSO with the desired amount of a browning mixture was poured on to 15 ml of hardened minimal media. After incubation for 48 hr in the dark, the colonies on the plates were counted using an Automated Colony Counter (New Brunswick Scientific Co., Edison, NJ). Both strains were examined for bacterial genetic properties prior to use. For each sample, dose-response assays were performed six times to ensure accurate results.

## RESULTS AND DISCUSSION

No mutagenic activity was evident from the spot tests of the browning mixtures obtained from neutral aqueous solution systems, and therefore, no dose-response assay of these samples was attempted.

Some reports have described variations in browning rate and types of compounds formed in terms of the reaction conditions, e.g. temperature, pH and initial reactants (Shibamoto & Bernhard, 1976). Spingarn & Garvie (1979) observed the effects of the antioxidant *n*-propyl gallate on the mutagenicity of products extracted from their model sugar–amine systems and found that the antioxidant inhibited the de-

velopment of mutagenic activity. Therefore, the commercial food antioxidants, BHA and BHT were added to the casein–lactose systems, to see whether these compounds had any similar effects on the mutagenic potency of the browning products. Sodium nitrite, a common food additive, was introduced into browning model systems containing glucose and cysteamine to study the effects of the presence of *N*-nitroso compounds in the Ames assay (Mihara & Shibamoto, 1980). However, the presence of nitrite and/or BHA and BHT did not affect the mutagenic potential of browning mixtures produced in the casein–lactose model browning system. The casein–lactose model system heated at pH 10 for 6 hr also produced compounds of insignificant mutagenic activity, as indicated in Table 1. Therefore, an increase in basicity in an aqueous system did not enhance either the number or concentration of potentially mutagenic compounds, as might have been expected from studies demonstrating the increase in rate of browning and in formation of products with increasing pH (Ellis, 1959; Lea & Hannan, 1949).

In contrast to these results with the aqueous browning systems, the same reactants heated in the dry state yielded products that exhibited very strong mutagenicity to strain TA98 (Fig. 1) but only with S-9 mix.

It is important to define the sample levels of free histidine that, if available to Ames strains of *S. typhimurium*, would lead to a misinterpretation of results. It was not possible to achieve a concentration of free histidine in the sample plates sufficient to generate a response by the bacteria, for two reasons. First, a methylene chloride extract of the browning reaction mixture was used for the experiments and any unreacted histidine in the browning sample would not have been extracted by this nonpolar solvent. This was confirmed by the lack of any Biuret reaction in any of the samples prepared. Also, histidine makes up 2·5% of the amino-nitrogen in casein (Eskin, Henderson & Townsend, 1971) and thus is present in very small quantities initially. Amino-nitrogen losses in casein–lactose systems, as demonstrated by Rao & Rao (1972), were estimated at 38% of the initial value after 20 min at 121°C. In the Ames assay, $10^{-7}$ mol histidine/plate is added to allow for growth of the first generation of bacteria plated. Estimating the loss of amino-nitrogen in heated casein mixtures at 38%, the amount of histidine in the sample would be 100 times less than $10^{-7}$ mole per plate if the sample were all casein at the highest level, and is therefore insignificant.

Samples Ia, IIIa, IVa, Va, and VIa each showed a significant dose-related response against TA98 with S-9 mix (Fig. 1a). On the other hand, a tar obtained from heated lactose (sample IIa) was not mutagenic. Commercial baking soda ($NaHCO_3$) was used as an additional ingredient because of its common presence in baked foods and because it increased pH. Sample VIa was strongly positive in strain TA98 with S-9 mix, with the 50-μg/plate level yielding 16·75 times the spontaneous reversion rate.

The presence of baking soda enhances the rate of formation of browning products. Yet an increase in pH from sample IIIa to sample IVa did not greatly enhance the mutagenic potential of the resultant products. Thus the presence of other substances, such as inorganic metals and nitrate, in commercially prepared non-fat dried milk may, in combination with the baking soda, account for the extreme mutagenicity of sample VIa. The non-fat dried milk samples Va and VIa also produced a response in strain TA100 with S-9 mix (Fig. 2) while other samples were sensitive only to strain TA98. This again points to some unknown augmenting factor in the non-fat dried milk samples. The presence of other proteins, such as lactalbumin and lactoglobulin, would also have an effect on the formation of browning products. It is possible, therefore, that in the presence of biologically active elements and other proteins besides casein, an increase in the quantity and/or diversity of products formed in the browning reactions would be evident.

Of the samples recovered from the Porapak Q traps, Ib, IVb, Vb and VIb were mutagenic toward strain TA98 with S-9 mix (Fig. 1b) but, the samples IIb and IIIb were not. This implies mutagenic activity in low-molecular-weight volatile compounds formed during the browning reaction in food. None of the samples from the Porapak traps showed any response to strain TA100 with or without S-9 mix.

The positive responses of TA98 with S-9 suggest the presence of frameshift mutagens requiring activation. Frameshift mutations are usually caused by heterocyclic compounds which are activated by epoxidation

Table 1. *The results of mutation tests on the browning reaction mixtures obtained from a casein–lactose model system heated at pH 10 and 100°C for 6 hr*

| Test concn (μg tar/plate) | No. of revertants/plate | | | |
|---|---|---|---|---|
| | Strain TA98 | | Strain TA100 | |
| | With S-9 | Without S-9 | With S-9 | Without S-9 |
| SR | 43 ± 12·5 | 27 ± 5·1 | 102 ± 14·1 | 165 ± 22·7 |
| 0 | 33 ± 11·2 | 23 ± 8·2 | 102 ± 8·1 | 145 ± 7·2 |
| 50 | 33 ± 1·2 | 35 ± 8·1 | 164 ± 6·4 | 113 ± 3·5 |
| 100 | 35 ± 3·1 | 32 ± 4·6 | 110 ± 7·8 | 183 ± 26·3 |
| 500 | toxic | 34 ± 13 | 90 ± 25·1 | 186 ± 12·1 |
| 1000 | toxic | 39 ± 2 | 72 ± 36·5 | 181 ± 12·1 |
| 1500 | toxic | 41 ± 6·2 | 105 ± 2·1 | 181 ± 19·9 |

SR = Spontaneous revertants

Values are means ± 1 SD of six replications.

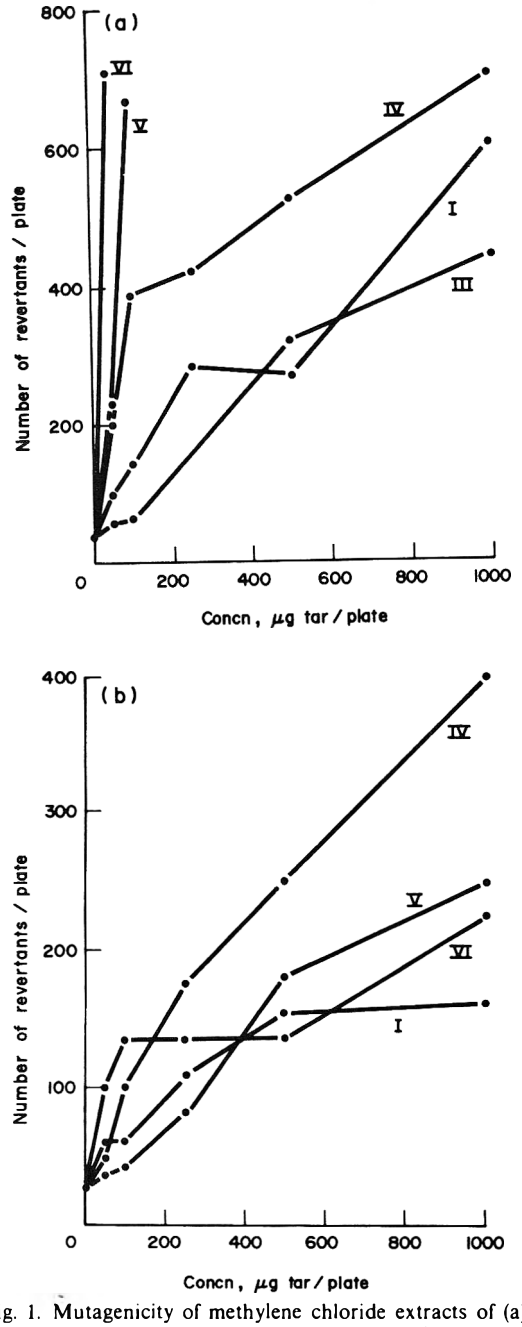


Fig. 1. Mutagenicity of methylene chloride extracts of (a) the residual tars and (b) the Porapak Q traps following the dry heating of (I) casein, (III) casein/lactose, (IV) casein/lactose/baking soda, (V) non-fat dried milk and (VI) non-fat dried milk/baking soda. Assays were carried out using *Salmonella typhimurium* TA98 with S-9 mix. Each point is the mean of six replications.

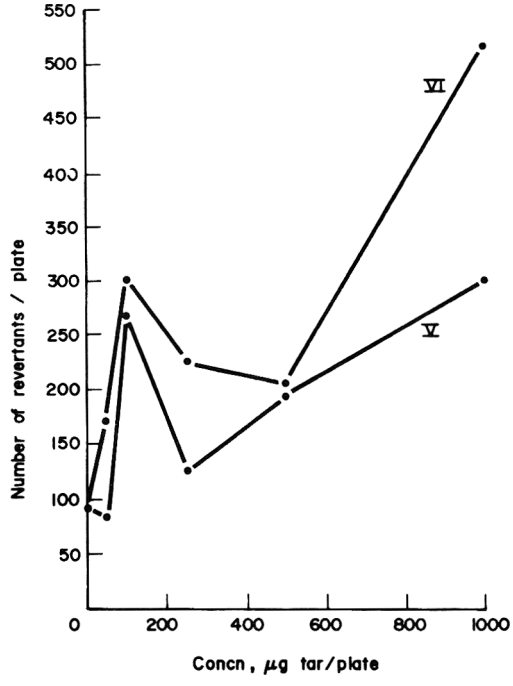


Fig. 2. Mutagenicity of methylene chloride extracts of the residual tars resulting from the dry heating of non-fat dried milk (V) alone or (VI) with baking soda, in assays against *Salmonella typhimurium* TA100 with S-9 mix. Each point is the mean of six replications.

and become covalently bound after intercalation into the tertiary structure of the DNA molecule. This causes physical separation of DNA nucleotides and damage during replication. Many nitrogen and oxygen heterocyclic compounds (e.g. aflatoxin) have been implicated as frameshift mutagens, and these types of compound have also been isolated and identified in model milk-browning systems (Ferretti *et al.* 1970; Shibamoto, Mihara, Nishimura *et al.* 1980).

The compounds trapped on porous polymer are usually very volatile chemicals of low molecular weight. Most of these chemicals generally escape from foods into the atmosphere during heat treatment. Consequently some mutagenic volatile chemicals formed during heat treatment may cause a long-term occupational health problem.

Since many foods are heated during preparation, it is important to understand the biological activities of the compounds produced. These compounds are commonly known to consumers in terms of their flavour or colour, yet it is becoming apparent that their properties extend beyond those that the senses perceive. Simple sugar–amine model systems have been shown to produce compounds with intense mutagenicity. Now, a more complex system containing the common food-ingredient milk has been shown to produce substances of potential hazard. The presence of mutagens or co-mutagens in simple and more complex browning mixtures demands further examination so that the hazard to the population can be defined more clearly and possibly circumvented.

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# THE MUTAGENICITY OF SOME EDIBLE MUSHROOMS IN THE AMES TEST

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**Abstract**—The mutagenic activity of five wild and two cultivated species of edible mushrooms was studied in the Ames Salmonella/microsome test system. The wild mushrooms tested were four species of the genus Lactarius (*L. necator*, *L. torminosus*, *L. helvus* and *L. rufus*) and bolete (*Boletus edulis*). The cultivated species were champignon (*Agaricus bisporus*) and shiitake (*Lentinus edodes*). All the mushrooms were mutagenic to tester strains sensitive to base-pair substitution mutagens, and *L. necator*, *L. rufus* and *B. edulis* also had some frameshift activity. Metabolic activation was not required and the mutagenic activity could be detected even in boiled mushroom extracts. After fractionation with organic solvents (ethanol followed by diethyl ether) the activity was recovered in the ether phase as well as the aqueous phase in the case of *L. necator*, but remained in the aqueous phase of the *A. bisporus* and *Lentinus edodes* extracts.

## INTRODUCTION

The mutagenic activity of some edible wild mushrooms of the genus Lactarius in the *Salmonella typhimurium* tester strains TA100 and TA98 was recently reported (Knuutinen & von Wright, 1982). Because of the relatively large and potentially increasing use of mushrooms as human food, it was considered necessary to retest these mushroom species using a more complete set of Ames tester strains and to include in the test programme mushrooms of other genera consumed more extensively by man. The additional species chosen for this study were bolete (*Boletus edulis*), a highly valued wild mushroom, together with champignon (*Agaricus bisporus*) and shiitake (*Lentinus edodes*), the two most important mushroom species in world trade.

In addition to direct mutagenicity tests, a study was made of the effect of boiling on the mutagenicity of *Lactarius necator*, *Boletus edulis*, *Agaricus bisporus* and *Lentinus edodes*. Furthermore, three of the species were extracted with organic solvents to fractionate the mutagenic agents.

## EXPERIMENTAL

*Test materials.* Samples of the Lactarius species investigated (*L. necator*, *L. torminosus*, *L. helvus* and *L. rufus*) were gathered in September 1980 in Southern Finland. *Agaricus bisporus* and *Lentinus edodes* were obtained from the experimental mushroom-cultivation plant of the Food Research Laboratory of the Technical Research Centre of Finland. Samples of *Boletus edulis* were kindly provided by Valio, the Finnish Co-operative Dairies' Association. All the mushrooms were stored at −20°C.

*Preparation of crude mushroom extracts.* The frozen mushrooms were homogenized in a Bamix M-100 household homogenizer. The homogenate was filtered and the filtrate was centrifuged to remove any remaining mushroom debris. The supernatant was sterilized by filtration through a Sartorius (0·45 μm) filter (Sartorius GmbH, Göttingen, FRG) and was used immediately for mutagenicity tests. About 60 ml mushroom extract was obtained from each 100 g of mushrooms.

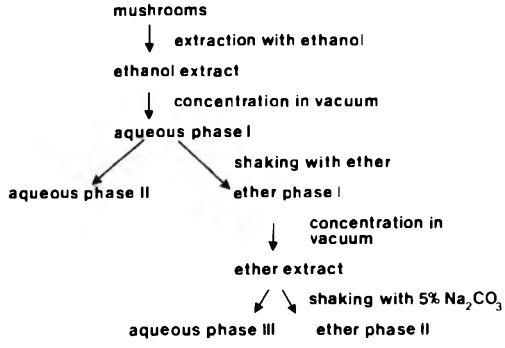


Fig. 1. Fractionation scheme for the mutagenic agents in mushrooms. Sliced mushrooms (1 kg) were incubated in 1 kg 99% ethanol at room temperature for 72 hr. The ethanol extract was collected, filtered and evaporated in a vacuum evaporator (Heidolph Elektro KG, Kelheim, FRG) to yield aqueous phase I (about 300 ml). This was shaken with an equal volume of diethyl ether, the phases were separated and each was concentrated in a vacuum to about 50 ml (aqueous phase II and ether phase I). Ether phase I was shaken with 100 ml 5% $Na_2CO_3$ solution to yield ether phase II and aqueous phase III. The latter was concentrated to half of the original volume and neutralized with 1 N-HCl before the mutagenicity tests.

*Mutagenicity tests.* The test procedure was that described by Ames, Durston, Yamasaki & Lee (1973). The tester strains were TA100 and TA98 (McCann, Spingarn, Kobori & Ames, 1975) and TA1535, TA1537 and TA1538 (Ames, Lee & Durston, 1973). The liver microsomal fraction (S-9 mix) was obtained from phenobarbital-induced male NMRI mice of 30 g body weight (supplied by Oy Orion Ab, Finland). Five dose levels of mushroom extract (10, 25, 50, 75 and 100 μl/plate) were used. Each dose was tested in

Table 1. *Mutagenic activities of the extracts of seven species of mushroom, determined by the Ames test*

| Mushroom species | Amount of mushroom extract (μl/plate) | No. of revertants/plate* in tester strain: TA100 −S-9 | TA100 +S-9 | TA98 −S-9 | TA98 +S-9 | TA1535 −S-9 | TA1535 +S-9 | TA1537 −S-9 | TA1537 +S-9 | TA1538† −S-9 | TA1538† +S-9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lactarius necator* | 0 | 71·0 | 75·3 | 10·3 | 19·7 | 40·3 | 17·0 | 2·3 | 2·7 | 11·7 | 20·0 |
| | 10 | 121·3 | 106·3 | 15·3 | 16·7 | 39·7 | 20·0 | 24·3 | 6·0 | 18·3 | 24·0 |
| | 25 | 239·0 | 123·0 | 20·0 | 19·0 | 54·0 | 19·3 | 70·7 | 9·7 | 15·7 | 12·0 |
| | 50 | 254·7 | 171·0 | 29·7 | 24·3 | 62·0 | 49·3 | 112·5 | 15·5 | 19·7 | 14·3 |
| | 75 | 386·0 | 345·0 | 37·5 | 30·0 | 81·0 | 40·3 | 148·5 | 49·7 | 16·0 | 13·0 |
| | 100 | 416·0 | 396·7 | 49·3 | 40·0 | 87·0 | 52·7 | 144·7 | 33·0 | 13·0 | 15·7 |
| *L. torminosus* | 0 | 108·0 | 112·0 | 17·7 | 21·0 | 30·7 | 18·0 | 4·3 | 5·5 | 11·7 | 20·0 |
| | 10 | 109·7 | 109·7 | 18·7 | 22·7 | 42·7 | 24·3 | 6·3 | 6·0 | 16·7 | 14·3 |
| | 25 | 128·7 | 128·0 | 15·7 | 22·7 | 51·3 | 33·7 | 5·3 | 3·3 | 15·7 | 9·0 |
| | 50 | 169·7 | 148·0 | 21·3 | 19·3 | 67·3 | 33·3 | 4·7 | 5·3 | 18·0 | 10·7 |
| | 75 | 178·0 | 182·7 | 19·0 | 18·0 | 51·0 | 33·7 | 3·7 | 1·7 | 13·0 | 11·7 |
| | 100 | 212·3 | 185·3 | 18·3 | 21·7 | 66·7 | 29·7 | 4·7 | 1·3 | 10·0 | 10·0 |
| *L. helvus* | 0 | 108·0 | 89·7 | 10·7 | 23·7 | 30·7 | 22·3 | 1·7 | 5·0 | 22·0 | 19·0 |
| | 10 | 114·7 | 126·3 | 14·0 | 16·7 | 48·0 | 34·3 | 1·3 | 3·0 | 18·5 | 22·0 |
| | 25 | 128·7 | 132·0 | 12·3 | 20·7 | 51·3 | 44·3 | 3·0 | 2·0 | 16·5 | 20·5 |
| | 50 | 152·3 | 156·0 | 12·7 | 21·0 | 149·7 | 145·3 | 1·0 | 1·0 | 13·5 | 16·5 |
| | 75 | 145·3 | 154·0 | 17·0 | 25·0 | 104·3 | 71·0 | 1·3 | 1·3 | 12·0 | 13·5 |
| | 100 | 232·0 | 193·7 | 15·7 | 21·7 | 77·3 | 42·0 | 0·3 | 1·0 | 12·0 | 11·0 |
| *L. rufus* | 0 | 75·7 | 78·3 | 13·3 | 16·7 | 30·0 | 20·3 | 4·3 | 3·7 | 18·3 | 26·7 |
| | 10 | 102·7 | 117·0 | 23·7 | 14·7 | 44·0 | 44·0 | 17·0 | 16·0 | 22·7 | 18·7 |
| | 25 | 156·7 | 123·7 | 30·7 | 20·0 | 66·0 | 78·7 | 36·7 | 35·7 | 25·0 | 15·3 |
| | 50 | 201·0 | 131·3 | 38·0 | 26·0 | 136·7 | 135·3 | 74·0 | 60·3 | 23·0 | 10·7 |
| | 75 | 213·0 | 149·3 | 71·0 | 22·7 | 187·0 | 183·0 | 82·3 | 78·0 | 27·7 | 6·0 |
| | 100 | 244·7 | 176·3 | 87·0 | 26·0 | 193·0 | 191·3 | 131·3 | 89·7 | 16·7 | 1·5 |
| *Boletus edulis* | 0 | 121·3 | 134·7 | 13·3 | 26·0 | 20·0 | 16·0 | 6·7 | 5·0 | 28·7 | 33·3 |
| | 10 | 127·7 | 131·0 | 19·0 | 28·3 | 25·7 | 13·7 | 3·7 | 7·5 | 20·7 | 24·7 |
| | 25 | 164·7 | 164·7 | 18·7 | 26·3 | 27·0 | 18·7 | 2·3 | 3·0 | 18·0 | 25·0 |
| | 50 | 212·3 | 192·0 | 25·3 | 33·3 | 22·0 | 21·7 | 6·0 | 5·3 | 20·7 | 19·7 |
| | 75 | 225·3 | 188·3 | 30·0 | 42·7 | 29·0 | 23·7 | 3·3 | 3·7 | 30·0 | 6·0 |
| | 100 | 261·7 | 230·0 | 44·7 | 49·7 | 42·3 | 29·3 | 5·7 | 8·0 | 25·3 | 4·7 |
| *Agaricus bisporus* | 0 | 111·7 | 117·0 | 12·0 | 21·0 | 36·0 | 25·3 | 5·7 | 4·3 | 17·7 | 25·3 |
| | 10 | 135·7 | 136·0 | 12·3 | 29·0 | 43·7 | 30·0 | 5·5 | 7·0 | 16·7 | 24·3 |
| | 25 | 162·7 | 139·3 | 7·9 | 20·5 | 58·3 | 28·3 | 3·0 | 5·0 | 15·3 | 22·7 |
| | 50 | 206·3 | 177·0 | 15·0 | 18·3 | 89·3 | 27·3 | 8·9 | 4·7 | 8·3 | 15·3 |
| | 75 | 231·3 | 241·7 | 20·0 | 16·7 | 111·5 | 34·3 | 4·0 | 4·7 | 2·7 | 7·3 |
| | 100 | 244·7 | 250·0 | 15·5 | 16·3 | 98·0 | 53·0 | 1·3 | 2·3 | 0·3 | 2·7 |
| *Lentinus edodes* | 0 | 117·7 | 134·0 | 15·3 | 24·7 | 29·0 | 24·7 | 4·3 | 7·0 | 24·3 | 34·0 |
| | 10 | 102·0 | 130·7 | 12·0 | 31·7 | 29·3 | 25·0 | 8·5 | 3·0 | 20·7 | 22·0 |
| | 25 | 126·0 | 147·3 | 9·3 | 28·3 | 25·3 | 22·0 | 3·7 | 4·0 | 15·7 | 18·3 |
| | 50 | 187·7 | 238·0 | 12·0 | 29·7 | 26·3 | 22·3 | 5·0 | 8·0 | 10·3 | 7·3 |
| | 75 | 257·3 | 253·0 | 15·0 | 36·7 | 31·0 | 27·0 | 3·0 | 8·3 | 10·3 | 5·5 |
| | 100 | 274·7 | 244·7 | 19·3 | 30·3 | 51·0 | 49·0 | 3·0 | 7·0 | 10·0 | 9·0 |

*Values are the means from three experiments.
†Some growth inhibition was observed in this strain with high doses of the mushroom extracts.

Table 2. *Fractionation patterns of the mutagenic activities of* Lactarius necator, Agaricus bisporus *and* Lentinus edodes *in tests on* Salmonella typhimurium *strain TA100*

| Mushroom species | Test fraction†... | No. of revertants/plate* with doses of 100 μl of fraction/plate: Aqueous phase II | Ether phase I | Aqueous phase III | Ether phase II |
|---|---|---|---|---|---|
| *L. necator* | | 165·0 ± 22·1 | 257·0 ± 22·1 | 198·0 ± 30·5 | 10·0 ± 7·8 |
| *A. bisporus* | | 193·1 ± 21·6 | 11·6 ± 5·9 | 1·2 ± 12·4 | 13·6 ± 22·6 |
| *Lentinus edodes* | | 236·0 ± 15·1 | 4·7 ± 7·4 | 0·6 ± 1·5 | 5·3 ± 10·4 |

*Values are means ± SD for three plates. The mean control value (86·0) has been subtracted.
†For identity of fractions see Fig. 1.

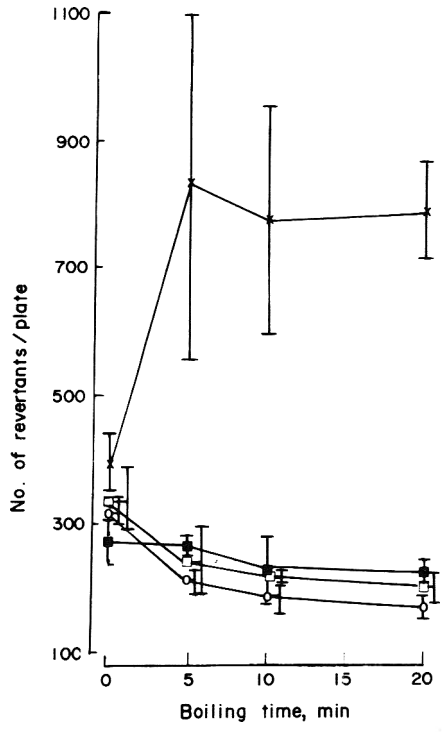


Fig. 2. Effect of boiling on the mutagenic activities of *Lactarius necator* (×), *Boletus edulis* (○), *Agaricus bisporus* (□) and *Lentinus edodes* (■) towards *Salmonella typhimurium* strain TA100. Each point represents the mean of two experiments (±SD).

triplicate plates with and without metabolic activation.

*Boiling experiments.* To simulate the effects of food processing, 10-ml samples of crude extracts of *L. necator*, *B. edulis*, *A. bisporus* and *Lentinus edodes* were placed in a boiling water bath. The samples, in 20-ml test tubes, were loosely covered. Aliquots of 100 μl were withdrawn after various periods of boiling and were tested for mutagenicity using the tester strain TA100 without metabolic activation.

*Fractionation of mutagenic activity. L. necator*, *A. bisporus* and *Lentinus edodes* were chosen for fractionation with organic solvents (ethanol and diethyl ether). The general fractionation scheme is shown in Fig. 1. The mutagenicity of the fractions was tested with strain TA100 without metabolic activation.

## RESULTS

All the mushroom species under investigation showed some mutagenic activity in the tester strains TA100 and TA1535, which are sensitive to base-pair substitution mutagens (Table 1). The frameshift tester strain TA98 was reverted by extracts of *L. necator*, *L. rufus* and *B. edulis*. *L. necator* and *L. rufus* extracts also gave positive results with another frameshift strain, TA1537. The presence of the S-9 metabolic activation system was not found to enhance the mutagenicity of any mushroom extract. Indeed with the frameshift tester strains particularly, the presence of the microsomal fraction somewhat reduced the mutagenic effects.

*L. necator* was by far the most mutagenic species (Table 1). This species not only maintained its mutagenic activity during the boiling experiment, but the activity actually doubled (Fig. 2). Boiling had no observable effect on the activity of *Lentinus edodes*, while the mutagenicity of *A. bisporus* and of *B. edulis* was reduced to about half that of the original extract within the first 10 min, remaining at that level until the end of the 20-min boiling period. It should be noted that the reduction in the volume of the mushroom extract during boiling was less than 10%.

In the fractionation of *L. necator*, mutagenic activity was detected in both the aqueous phase II and the ether phase I (Fig. 1), but was more marked in the latter (Table 2). After the ether extract had been washed with 5% $Na_2CO_3$ solution, the mutagenicity was totally recovered in the aqueous phase III. With *A. bisporus* and *Lentinus edodes* the mutagenic agent(s) remained in the aqueous phase II after the first ether extraction.

## DISCUSSION

These results indicate that mutagenic compounds occur widely among the Basidiomycetes even in taxonomically unrelated species. The chemical nature of these compounds is at present unknown. Their different tester-strain specificities, together with different thermolabilities and fractionation patterns, strongly suggest that they differ structurally.

The significance of these findings to human health is still a matter of speculation. Only after the mutagenic compounds in the mushrooms have been properly isolated and characterized can anything more specific be stated. It is possible that these compounds are identical to or resemble some known chemicals for which carcinogenicity data are well established. This would, of course, make the risk assessment relatively easy. On the other hand, it is possible that the compounds present a novel group of chemicals, which may be difficult to synthesize. In this case only long-term animal studies would give any reliable indication of the risks involved in consumption of the mushrooms. We are attempting to isolate and characterize the compounds in question.

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# NITRATE REDUCTASE ACTIVITY AND NITRITE LEVELS IN THE SALIVA OF HABITUAL USERS OF VARIOUS TOBACCO PRODUCTS

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**Abstract**—Nitrite found in human saliva is the product of the microbial reduction of nitrate circulating through the salivary glands. Saliva samples were collected under controlled conditions from volunteers who were habitual users of different types of tobacco products (tobacco chewers, cigarette smokers, bidi smokers and masheri users) and from controls. The saliva samples were analysed for nitrite levels and for nitrate reductase activity spectrophotometrically. Samples were collected from two different areas, Bombay (urban) and Ghodegaon (rural). Salivary nitrite levels in the control groups ranged from 1·6 ppm in Ghodegaon to 11 ppm in Bombay. The nitrite levels of the masheri-using groups from both locations and of the tobacco-chewing group from Bombay were significantly higher than those of the controls. A number of volunteers showed undetectable levels of salivary nitrate reductase. Volunteers who chewed tobacco or used masheri had higher levels of nitrate reductase activity than the controls. However, there did not seem to be any clear correlation between nitrite levels and nitrate reductase activity in the saliva.

## INTRODUCTION

Saliva taken directly from the salivary gland ducts does not contain nitrite (Savostianov, 1937; Tannenbaum, Sinskey, Weisman & Bishop, 1974). However, nitrites are present in saliva in the oral cavity and it has been shown that they are formed from nitrates which are released from the salivary ducts and are reduced by the action of nitrate reductase secreted by bacteria in the oral cavity (Goaz & Biswell, 1961). Recently concern has focused on the importance of nitrites as precursors of *N*-nitroso compounds.

A number of studies have been made in an attempt to identify the high-risk factors associated with oral cancer (Paymaster, 1962; Pindborg, Barones & Roed-Peterson, 1968). The suspected factors include smoking and tobacco chewing. Tobacco is rich in nitrates and secondary amines and we thought it important to study nitrite levels in the saliva of tobacco chewers and smokers. The present study relates to the variations in levels of salivary nitrite and nitrate reductase among groups of users of four types of tobacco product. These groups were habitual tobacco chewers, bidi smokers, cigarette smokers or masheri users. Bidi is a cheap cigarette, 4–7 cm long, made by rolling about 2 g of coarse tobacco in a dry timburni (*Diospysos melanoxylum*) leaf. Masheri is half-burnt tobacco, powdered and used as a powder for cleaning teeth. However, women in rural areas use it frequently and keep it under the gum in lieu of chewing tobacco.

*Abbreviations*: FMN = Flavin mononucleotide; NADPH = Reduced nicotinamide adenine dinucleotide phosphate.

## EXPERIMENTAL

*Chemicals.* FMN and NADPH were obtained from Sigma Chemical Co., St. Louis, MO, USA. *N*-(1-Naphthyl)ethylenediamine dihydrochloride was obtained from E. Merck, Darmstadt, FRG. All other chemicals were of analytical grade.

Nitrite concentration was determined by the classical Griess reaction with sulphanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride in an acid medium (Nicholas & Nason, 1957).

*Enzyme assay.* Nitrate reductase activity was measured by estimating the nitrite formed in the reaction mixture by the method of Nason & Evans (1956). The reaction system (0·05 ml) contained the following components: 27 μmol pyrophosphate buffer (pH 7·0), 20 μg FMN, 0·08 μmol NADPH, 10 μmol $KNO_3$ and an appropriate amount of saliva. After 5 min incubation at 35°C the reaction was stopped by adding 0·5 ml of 1% sulphanilamide in 25% (v/v) HCl. The resulting nitrite was measured colorimetrically. One unit of nitrate reductase was defined as that amount of enzyme that resulted in the formation of $10^{-3}$ μmol nitrite under the given conditions of assay.

*Test personnel.* Volunteers who participated in the present study were from two locations, a rural area, Ghodegaon, 200 km south-west of Bombay and Bombay itself. The subjects were males and females aged between 20 and 50 yr. Those who reported using more than one of the tobacco products under study were excluded so that the results and conclusions would reflect only individual habits of tobacco use.

*Collection of saliva.* The donors were asked to rinse their mouths with water and then expectorate for 15–20 min into wide-mouthed plastics bottles until

Table 1. *Levels of nitrite and nitrate reductase activity in saliva from habitual users of various tobacco products and controls*

| Tobacco-using habit | Salivary nitrite (ppm) | No. of volunteers (percentage) with no detectable nitrate reductase activity in saliva | Nitrate reductase activity (U/ml saliva)† |
|---|---|---|---|
| | **Bombay (urban) volunteers** | | |
| None (controls) | 11·1 ± 2·9 (33) | 16/33 (48) | 58·30 ± 6·70 (17) |
| Tobacco chewing | 20·4 ± 4·4* (16) | 5/16 (31) | 81·05 ± 15·00 (11) |
| Cigarette smoking | 8·9 ± 1·3 (16) | 3/16 (18) | 62·00 ± 13·00 (13) |
| Bidi smoking | 16·7 ± 3·0 (35) | 4/16 (25) | 70·00 ± 9·00 (12) |
| Masheri use | 19·8 ± 2·2* (9) | 0/9 (0) | 95·00 ± 12·00 (9) |
| | **Ghodegaon (rural) volunteers** | | |
| None (controls) | 1·6 ± 0·39 (13) | 7/13 (54) | 37·00 ± 7·00 (6) |
| Tobacco chewing | 1·7 ± 0·13 (19) | 12/19 (63) | 37·00 ± 7·34 (7) |
| Cigarette smoking | ND | 3/4 (75) | ND |
| Bidi smoking | ND | ND | ND |
| Masheri use | 6·02 ± 1·06* (13) | 2/13 (15) | 70·00 ± 12·00* (11) |

ND = Not determined

†Values are means ± SEM for the number of samples indicated in brackets and those marked with asterisks are significantly different (Student's *t*-test) from the corresponding control values ($P < 0·05$).

15–20 ml sample had been collected. The bottles had screw caps and contained 0·1 ml 0·1 N-NaOH to inhibit any bacterial growth. No attempt was made to separate any exfoliate cells either by centrifugation or filtration, since the aim was to test the nitrite and nitrate reductase levels in the naturally occurring milieu of the oral cavity. Nitrate reductase activity was determined immediately after collection in the saliva of ten volunteers in both the presence and absence of NaOH, and it was concluded that the presence of NaOH had no effect on enzyme activity. The nitrate reductase activity of saliva samples were also measured immediately after collection and 5-hr storage so as to determine that no enzyme activity would be lost during the transport of the samples from Ghodegaon to Bombay.

## RESULTS

Table 1 shows the amount of nitrite in the saliva of volunteers with different habits of tobacco use from Bombay and Ghodegaon. The nitrite levels of volunteers from Bombay, whether or not they used tobacco products, were higher than those of corresponding groups from Ghodegaon. It is also evident that among the Bombay volunteers the salivary nitrite levels of tobacco chewers and masheri users were twice those of non-users of tobacco products. Neither cigarette nor bidi smoking had any significant effect on nitrite levels in the saliva of volunteers from Bombay.

In Ghodegaon volunteers tobacco chewing did not affect salivary nitrite levels, but the use of masheri significantly increased the nitrite content of the saliva. The levels in both groups were much lower than those observed in the corresponding groups from Bombay.

The nitrate reductase activity of the bacterial flora in the oral cavity is indicative of oral hygiene and is responsible for the formation of nitrite from nitrates in the oral cavity. It was interesting to observe that a number of volunteers from virtually all of the groups had undetectable enzyme activity (Table 1). Among the Bombay volunteers, the percentage of people with no enzyme activity decreased with tobacco chewing, and cigarette and bidi smoking in comparison with the controls, and none of the masheri-using volunteers showed undetectable enzyme activity.

Among Ghodegaon volunteers the percentage of people with no detectable nitrate reductase activity was increased in cigarette and bidi smokers compared

Table 2. *Levels of nitrite and nitrate reductase activity in saliva of habitual tobacco chewers living in Bombay*

| Volunteer no.* | Age (yr) | Nitrite (ppm) | Nitrate reductase activity (U/mol saliva) |
|---|---|---|---|
| A5 | 30 | 20·5 | 108 |
| A6 | 30 | 10·8 | 60 |
| A7 | 30 | 17·2 | 56 |
| A8 | 35 | 43·8 | 18 |
| B8 | 35 | 5·2 | UD |
| D6 | 41 | 23·8 | 134 |
| D7 | 35 | 6·6 | 192 |
| E11 | 42 | 22·5 | UD |
| F7 | 29 | 9·6 | 33 |
| F9 | 26 | 6·6 | 58 |

UD = Undetectable

*All of the volunteers were male.

with the controls and was decreased remarkably only in masheri users. These observations indicate that oral hygiene is better in rural than in urban dwellers. However, the numbers of volunteers in the various groups are small and it is rather premature to draw any conclusions. However these observations serve as pointers for areas of future study.

Table 1 shows the mean values for nitrate reductase activity in the saliva of groups of volunteers with different habits of tobacco use who had measurable enzyme activity. Tobacco chewers, masheri users and controls from Bombay had much higher enzyme activity in their saliva than those in the corresponding groups from Ghodegaon. However, these differences were not significant statistically. Amongst volunteers from Bombay, those who used tobacco products had higher salivary enzyme activities than the controls, who did not use tobacco, but the differences were not statistically significant. Amongst Ghodegaon volunteers the enzyme activity in the masheri-using group was significantly higher than that in the control and tobacco-chewing group.

We then investigated whether there was any correlation between salivary nitrate reductase activity and the nitrite content of the saliva. Table 2 shows these values for Bombay volunteers who chewed tobacco. There was a wide variation between subjects in salivary nitrite levels (range 5·2–43·8 ppm). It is also evident that volunteers with undetectable or low enzyme activity do not have low nitrite values or *vice versa*. Nitrite levels in saliva are the result of two factors: (i) the intake of nitrate-rich food and (ii) the activity of nitrate reductase. It therefore appears that very low levels of enzyme activity which are undetectable by colorimetric methods are still sufficient to reduce nitrate to nitrite. It is also clear that there is no correlation between high enzyme activity and high nitrite levels in the saliva.

## DISCUSSION

These data clearly show that nitrite levels in the saliva of Bombay volunteers who did not use tobacco products are of the same order as those reported earlier (Shivapurkar, D'Souza & Bhide, 1980). However, it is very interesting to observe that the controls from Ghodegaon had extremely low salivary nitrite levels, and that these levels are even less than those reported by Tannenbaum *et al.* (1974). It is very difficult to conclude whether these low values are a reflection of low or undetectable nitrate reductase activity in the saliva of Ghodegaon volunteers or due to the low intake of nitrate-rich vegetables. Rural Indian populations generally subsist mainly on grains, legumes poor in nitrates and a few vegetables that grow in the vicinity. The above data clearly show the wide range in salivary nitrite levels from 1·6 in a rural area to 11·1 in an urban area. A survey carried out in Japan by Okabe (1973) determined salivary nitrite levels in 200 individuals and also showed a wide variation in nitrite values, which ranged from 0 to 200 ppm.

In contrast to our earlier observations on a small group of tobacco chewers (Shivapurkar *et al.* 1980), in the present study the salivary nitrite levels of the Bombay tobacco-chewing group were increased compared with the controls. Tobacco chewing did not affect salivary nitrate levels in the Ghodegaon group. On the other hand, the group of masheri-users from Ghodegaon had considerably higher nitrite levels as well as higher salivary nitrate reductase activity compared with the control group from the same area. This is likely to be so since frequent use of masheri and keeping it close to the gums has an adverse effect on oral hygiene, whereas tobacco is chewed and spat out within 30 minutes or so.

However, the lack of correlation between nitrate reductase activity and nitrite levels in samples of saliva indicate that although the bacterial enzyme activity may be one of the factors governing the dynamic equilibrium of salivary nitrite, it is not wholly responsible for the nitrite levels in saliva.

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# REDUCTION OF TERATOGENIC EFFECTS OF ETHYLENETHIOUREA IN RATS BY INTERACTION WITH SODIUM NITRITE *IN VIVO*

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**Abstract**—Nitrites are present in a wide variety of foods and their daily intake in man has been estimated at 1·5 mg. Ethylenethiourea (ETU), a major food contaminant resulting from degradation of ethylenebisdithiocarbamate fungicides, is a potent rat teratogen. The co-administration of ETU (60 or 40 mg/kg) and $NaNO_2$ (80 mg/kg) to rats by gavage on day 15 of gestation resulted in a higher survival of progeny than occurred with the corresponding dose of ETU alone. In a second study, ETU (60 mg/kg) and $NaNO_2$ (80, 100 or 120 mg/kg) were administered, either individually or in combination, as a single dose on day 13 of gestation. Administered alone, $NaNO_2$ did not produce any teratogenic response in full-term foetuses, whereas ETU produced a high incidence of various anomalies. However, the combined dosing resulted in the elimination of almost all the anomalies. The reducing effect of $NaNO_2$ on ETU-induced malformations was reversed when the animals were pretreated with 200 mg ascorbic acid/kg or 360 mg sodium ascorbate/kg. Since both of these are well-known inhibitors of *N*-nitrosation reactions, it was presumed that the simultaneous oral dosing of ETU and $NaNO_2$ resulted in the formation of *N*-nitrosoethylenethiourea.

## INTRODUCTION

Nitrates and nitrites are commonly found in foods. Nitrates, used as preservatives in meat and present in certain water supplies, are reduced to nitrites by plant enzymes and micro-organisms usually present in foods, intestinal contents and saliva (Hawksworth & Hill, 1971; Tannenbaum, Sinskey, Weisman & Bishop, 1974). The average human intake has been estimated to be about 1·5 mg/day for nitrites (Sander, 1967) and 400–500 mg/wk for nitrates (UNEP/WHO, 1978). Currently, allowable levels of nitrite added to foods range from 150 to 200 ppm (Olajos & Coulston, 1978).

A number of pesticides have been reported to undergo nitrosation in the presence of sodium nitrite in the stomach or *in vitro* under conditions simulating those in the stomach. Included among these are ziram, carbaryl, benzthiazuron, propoxur, triazine and ferbam (Lijinsky & Schmähl, 1978; Olajos & Coulston, 1978). Many of their nitroso derivatives have been reported to possess carcinogenic properties (Lijinsky & Schmähl, 1978), but only a few nitroso compounds have been reported to have anatomic teratogenic activity. Those found to be teratogenic were nitrosoethylurea (Druckrey, Ivankovic & Preussmann, 1966), nitrosomethylurea (Koyama, Handa, Handa & Matsumoto, 1970), nitrosopropylurea, nitrosomethylaniline and nitrosoethylaniline (Alexandrov, 1973).

Ethylenethiourea (ETU), a degradation and metabolic product of the ethylenebisdithiocarbamate group of fungicides, is frequently present on vegetables (Pecka, Baulu & Newsome, 1975); its concentrations tend to increase during cooking (Newsome & Laver, 1973) or during commercial processing as a result of degradation of the parent chemical (Phillips, Grady & Freudenthal, 1977). It has been reported that when given simultaneously with sodium nitrite by the oral route to the mouse, ETU acquired mutagenic properties (Seiler, 1975) and teratogenic properties (Teramoto, Saito & Shirasu, 1980), purportedly because of the formation of *N*-nitrosoethylenethiourea (Seiler, 1975).

Our studies have shown that both post- and prenatal adverse effects of ETU were eliminated, for the most part, when ETU was administered orally together with $NaNO_2$. However, the adverse effects were not eliminated when the combined ETU–$NaNO_2$ administration was immediately preceded by an oral dose of ascorbic acid or sodium ascorbate, both of which are well known as inhibitors of the formation of *N*-nitroso compounds *in vivo* from nitrosatable precursors. The results of these studies are reported here.

## EXPERIMENTAL

*Chemicals.* ETU (2-imidazolidinethione) was recrystallized to >99% purity as determined by thin-layer chromatography and melting point (Ruddick & Khera, 1975). Sodium nitrite ($NaNO_2$) and L-ascorbic acid (LAA), both AnalaR grade, were from British Drug Houses, Poole, Dorset, UK. Sodium ascorbate (NaA), USP–FCC, was obtained from Hoffman-LaRoche, Montreal. All chemicals were administered in aqueous solution in volumes of 1 ml/200 g body weight at appropriate concentrations.

*Animals.* Virgin female rats of 175–200 g body weight were paired overnight with proven breeders. The morning that a positive vaginal smear was observed was noted as day 1 of pregnancy. Feed (Master Feed, Master Feed Mills Ltd, Prescott, Ontario or Purina Lab Chow, certified, from Ralston-Purina Co.

Table 1. *Postnatal effects of combined oral administration of ethylenethiourea (ETU) and sodium nitrite to rats on day 15 of pregnancy*

| Treatment (mg/kg) | No. of litters | No. of pups: At birth | No. of pups: Dying postnatally | No. of pups: With hydrocephalus* |
|---|---|---|---|---|
| ETU (60) + $NaNO_2$ (80) | 3 | 40 | 40 | 10/19 |
| ETU (60) | 5 | 47 | 47 | 38/43 |
| ETU (40) + $NaNO_2$ (80) | 4 | 45 | 14 | 0/10 |
| ETU (40) | 4 | 46 | 45 | 14/18 |
| $NaNO_2$ (80) | 4 | 46 | 15 | 0/3 |
| Distilled water (vehicle) | 3 | 38 | 8 | 0/2 |

*No. affected/no. examined.

Inc., St. Louis, MO) and water were available at all times except for a period extending from 1 hr before to 1 hr after the intubation of the test agents.

*Time of treatment.* Treatment was given on day 15 of pregnancy for the postnatal and day 13 for the prenatal studies. These days were found to be optimal for teratogenic effects at low doses of ETU in preliminary trials.

*Postnatal study (experiment 1).* Five to twelve mated females were randomly assigned to each experimental group. On day 15 of gestation, two groups of rats were given an orally intubated dose of 40 or 60 mg ETU/kg, followed immediately by a dose of 80 mg $NaNO_2$/kg. Four additional groups were given either 40 or 60 mg ETU/kg or 80 mg $NaNO_2$/kg or two successive doses of distilled water. Thereafter all groups were treated similarly. The dams were allowed to deliver normally and progeny were examined at birth and frequently thereafter, the animals being maintained for 140 days to assess survival and development. Pups that died while on test were autopsied to determine the cause of death.

*Prenatal studies (experiments 2 and 3).* In these experiments all test chemicals were administered to dams (12–17 dams/group) orally on day 13 of gestation. All the dams were autopsied on the last day of pregnancy. Uteri were searched for resorptions, and for dead and live foetuses. All live foetuses were weighed and examined for external malformations, and after appropriate processing, two-thirds of them were studied for gross visceral changes and the remainder for skeletal anomalies using standard procedures.

In experiment 2, the effects of $NaNO_2$ on the ETU-induced teratogenicity were assessed. An oral dose of 60 mg ETU/kg was followed immediately by an oral dose of 80, 100 or 120 mg $NaNO_2$/kg. Control groups received either 60 mg ETU/kg or 100 or 120 mg $NaNO_2$/kg, or distilled water (the vehicle) alone.

Effects of oral pretreatment with LAA or NaA on the ETU–$NaNO_2$ interaction were evaluated in experiment 3. Doses of 200 mg LAA/kg or 360 mg NaA/kg were followed immediately by either 60 mg ETU/kg and then 80 mg $NaNO_2$/kg or by each of the last two drugs individually or by distilled water only. Four further groups were pretreated with distilled water, followed immediately by 60 mg ETU/kg, by 80 mg $NaNO_2$/kg, by both or by distilled water.

*Statistical analysis.* Data from the postnatal study (Table 1), being limited to fewer litters per group, were not analysed. From maternal and foetal body-weight data from the prenatal studies the means and standard errors were calculated for each experimental group. Values of $t$ were calculated for test and control group differences in the means. Foetal anomalies were analysed individually with the litter as the basic unit. The proportion ($p$) of a litter having a particular anomaly was calculated and transformed to a normally distributed variable arc sin/$p$ value (Davies, 1954). The means and standard errors of these values for various groups were then derived (Hald, 1952). The $t$ test was used for intergroup comparisons. In experiment 2 (Table 2), the group given 60 mg ETU/kg was compared with the groups given either any one dose of $NaNO_2$ plus a similar dose of ETU or $NaNO_2$ (without ETU) or distilled water. In experiment 3 (Table 3) with 12 experimental groups, seven paired intergroup comparisons (i.e. all those that were relevant) were made. Intergroup differences significant at $P < 0.05$ are reported.

## RESULTS

### *Postnatal study (experiment 1)*

No treatment-related adverse effects were observed on pregnancy or parturition in any test or control group. The mean pup body weight at birth was reduced in the groups given 60 mg ETU/kg or 60 mg ETU/kg plus 80 mg $NaNO_2$/kg, but after 10 days of age the reduction in body weight ceased to exist. The group given 60 mg ETU/kg plus 80 mg $NaNO_2$/kg survived for a longer period than that given 60 mg ETU/kg alone (Fig. 1a). The survival time and survival index (pups alive at the end of the experiment versus the total number born) in the group given combined 40 mg ETU/kg and 80 mg $NaNO_2$/kg were higher than those in the 40-mg ETU/kg group (Fig. 1b). Many of the pups died within 40 days of birth and at autopsy the incidence of hydrocephalus in the (ETU plus $NaNO_2$)-treated groups was lower than that in the ETU groups (Table 1).

### *Prenatal studies*

In the second experiment, designed to assess the effect of $NaNO_2$ treatment on the teratogenicity of ETU, two of the 17 dams in the group given distilled water plus 120 mg $NaNO_2$/kg died. Periodic weight measurements during pregnancy failed to show any significant treatment-related effects. The foetal study revealed that the teratogenic effects of 60 mg ETU/kg

Table 2. *Foetal effects of administration of sodium nitrite and/or ethylenethiourea (ETU) to rats on day 13 of pregnancy*

| Parameter | Data for groups treated with single doses (mg kg)† of: ETU (60) + $NaNO_2$ (120) | ETU (60) + $NaNO_2$ (100) | ETU (60) + $NaNO_2$ (80) | ETU (60) + $H_2O$ | $H_2O$ + $NaNO_2$ (120) | $H_2O$ + $NaNO_2$ (100) | $H_2O$ (two doses) |
|---|---|---|---|---|---|---|---|
| No. of dams at term | 10 | 16 | 14 | 12 | 15 | 14 | 12 |
| Mean no. of live foetuses litter | 10·1 | 9·6 | 12·4 | 12·6 | 12·0 | 10·1 | 11·3 |
| Resorptions + dead foetuses (% of total implants) | 24* | 10 | 9 | 5 | 12 | 7 | 6 |
| Mean foetal weight (g) ± SEM | 4·4 ± 0·07 | 4·4 ± 0·05 | 4·4 ± 0·03 | 4·1 ± 0·03 | 4·6 ± 0·03 | 4·5 ± 0·03 | 4·6 ± 0·04 |
| Incidence of anomalies‡: | | | | | | | |
| Brachygnathia | 0 | 0 | 0 | 8 | 0 | 0 | 0 |
| Exencephaly | 0 | 0 | 0 | 28 | 0 | 0 | 0 |
| Hydrocephaly | 0 | 0 | 3 | 87 | 0 | 0 | 0 |
| Scoliosis | 0 | 0 | 0 | 25 | 0 | 0 | 0 |
| Hypoplastic cerebellum | 0 | 0 | 3 | 70 | 0 | 0 | 0 |
| Short tail | 0 | 0 | 0 | 95 | 0 | 0 | 0 |
| Forelimb: | | | | | | | |
| Micromelia | 0 | 0 | 0 | 85 | 0 | 0 | 0 |
| Ectrosyndactyly | 0 | 0 | 0 | 11 | 0 | 0 | 0 |
| Hindlimb talipes | 0 | 0 | 0 | 19 | 0 | 0 | 0 |

†All test doses were administered in aqueous solution in a volume of 1 ml/200 g body weight. Distilled water was administered in the same volume.
‡(No. of foetuses affected/total examined) × 100.
The value marked with an asterisk differs significantly ($P < 0·05$) from the value for the distilled-water control.

were markedly reduced by the simultaneous 80-mg $NaNO_2$/kg dose and were completely abolished by 100 and 120 mg $NaNO_2$/kg (Table 2). Several foetal anomalies, not listed in Table 2, occurred at low frequencies and were unrelated to any particular treatment. These included retarded ossifications of the calvarium, centra and sternebrae, supernumerary rib, wavy rib and dilatation of the renal pelvis. Mean foetal weight was reduced in the 60-mg ETU/kg group but the difference from the control (distilled water) was not statistically significant. Compared with the control values, the mean number of live foetuses per litter was lower in the group given 60 mg ETU/kg with 100 mg $NaNO_2$/kg and the number of resorptions plus dead foetuses was higher in the group given 60 mg ETU/kg with 120 mg $NaNO_2$/kg. Only the latter difference was statistically significant (Table 2).

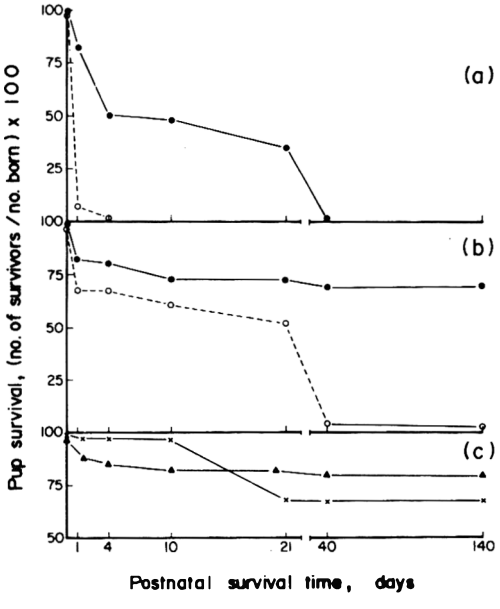


Fig. 1. Survival of progeny derived from rats given (a) 60 mg ETU/kg (○) or 60 mg ETU/kg + 80 mg $NaNO_2$ (●), (b) 40 mg ETU/kg (○) or 40 mg ETU/kg + 80 mg $NaNO_2$/kg (●) and (c) 80 mg $NaNO_2$/kg (×) or distilled water (▲).

In experiment 3, the treatments with 200 mg LAA/kg or 360 mg NaA/kg were tested to ascertain their effect on the ETU–$NaNO_2$ interaction (Table 3). The occurrences of foetal anomalies and the foetal-weight reduction in the groups given LAA or NaA with ETU and $NaNO_2$ (groups 1 and 6) were similar to those in the positive control groups given 60 mg ETU/kg alone or in combination with LAA or NaA (groups 3, 2 and 7, respectively). Negative control groups treated with LAA or NaA either alone or together with $NaNO_2$ (groups 5, 9, 4 and 8) or even the group treated with 60 mg ETU/kg and 80 mg $NaNO_2$/kg (group 10) failed to show any important teratogenic effect or reduction in foetal weight. A low incidence of foetal aberrations similar to those observed in experiment 2 and unrelated to any treatment was also noticed; these are not shown in Table 3. It was apparent that LAA and NaA at the dose levels given reversed the 'protective' effect exercised by $NaNO_2$ on the teratogenicity of ETU observed in experiment 2. These findings were readily reproduced in a replicate experiment (data not reported).

## DISCUSSION

In the studies reported here, it became quite clear that the teratogenicity of ETU ceased to manifest itself when oral administration of 60 mg ETU/kg was at once followed by an oral dose of 80–120 mg $NaNO_2$/kg. This 'protective' effect, however, was easily reversed by an oral pretreatment with LAA (200 mg/kg) or NaA (360 mg/kg). It has long been established that these two compounds can inhibit the formation of *N*-nitroso derivatives (Greenblatt, 1973; Kamm, Dashman, Conney & Burns, 1973; Mirvish, Wallcave, Eagen & Shubik, 1972). The mechanism of this inhibition was considered to be due to the rapid reaction, under acidic conditions, of ascorbic acid or ascorbate ion with nitrite to produce nitric oxide and dehydroascorbic acid (Dahn, Loewe & Bunton, 1960;

Table 3. *Prenatal effects of ascorbic acid (LAA) or sodium ascorbate (NaA) administered in combination with ethylenethiourea (ETU) and/or sodium nitrite to rats on day 13 of pregnancy*

| | | Data for rats treated with single doses (mg/kg)† of: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Group no. | LAA (200) + ETU (60) + $NaNO_2$ (80) | LAA (200) + ETU (60) + $H_2O$ | $H_2O$ + ETU (60) + $H_2O$ | LAA (200) + $H_2O$ + $NaNO_2$ (80) | LAA (200) + $H_2O$ × 2 | NaA (360) + ETU (60) + $NaNO_2$ (80) | NaA (360) + ETU (60) + $H_2O$ | NaA (360) + $H_2O$ + $NaNO_2$ | NaA (360) + $H_2O$ × 2 | $H_2O$ + ETU (60) + $NaNO_2$ (80) | $H_2O$ × 2 + $NaNO_2$ (80) | $H_2O$ × 3 |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| No. of dams at term | | 12 | 12 | 13 | 12 | 12 | 13 | 12 | 12 | 12 | 14 | 14 | 12 |
| No. of live foetuses litter (mean ± SEM) | | 9·4 ± 0·9 | 9·3 ± 0·6 | 10·5 ± 0·6 | 10·3 ± 0·7 | 9·8 ± 0·5 | 10·4 ± 0·5 | 10·0 ± 0·6 | 9·9 ± 0·6 | 9·3 ± 0·6 | 10·1 ± 0·3 | 9·7 ± 0·5 | 10·4 ± 0·7 |
| Resorptions + dead foetuses (% of total implants) | | 3 | 9 | 8 | 5 | 9 | 3 | 6 | 8 | 7 | 10 | 5 | 3 |
| Mean foetal weight (g) ± SEM | | 4·1 ± 0·04 | 4·0 ± 0·03 | 4·1 ± 0·03 | 4·7 ± 0·03 | 4·7 ± 0·04 | 3·9 ± 0·03 | 4·0 ± 0·03 | 4·8 ± 0·05 | 4·6 ± 0·04 | 4·5 ± 0·03 | 4·8 ± 0·02 | 4·9 ± 0·03 |
| Incidence of anomalies‡ | | | | | | | | | | | | | |
| Cleft palate | | 0 | 0 | 0 | 0 | 0 | 2 | 8§ | 0 | 0 | 0 | 0 | 0 |
| Brachygnathia | | 17 | 16 | 14 | 0 | 0 | 15 | 15 | 0 | 0 | 0 | 0 | 0 |
| Exencephaly | | 70 | 62 | 63 | 0 | 0 | 60 | 42 | 0 | 0 | 0 | 0 | 0 |
| Hydrocephaly | | 90 | 88 | 97 | 0 | 0 | 90 | 90 | 0 | 0 | 1 | 0 | 0 |
| Scoliosis | | 27 | 26 | 30 | 0 | 0 | 38 | 29 | 0 | 0 | 0 | 0 | 0 |
| Hypoplastic cerebellum | | 90 | 88 | 100 | 0 | 0 | 71 | 70 | 0 | 0 | 0 | 0 | 0 |
| Short tail | | 93 | 98 | 98 | 0 | 0 | 87 | 98 | 0 | 0 | 1 | 0 | 0 |
| Forelimb: | | | | | | | | | | | | | |
| Micromelia | | 88 | 91 | 100 | 0 | 0 | 77 | 93 | 0 | 0 | 0 | 0 | 0 |
| Ectrosyndactyly | | 23 | 7 | 4 | 0 | 0 | 22 | 18 | 0 | 0 | 0 | 0 | 0 |
| Hindlimb talipes | | 27 | 24 | 20 | 0 | 0 | 16 | 16 | 0 | 0 | 0 | 0 | 0 |

†All test doses were administered in aqueous solution in a volume of 1 ml/200 g body weight. Distilled water was administered in the same volume.
‡(No. of foetuses affected/total examined) × 100.
§Lower lip was fused with the mandible in one foetus.

Fan & Tannenbaum, 1973). Thus the formation of *N*-nitroso compounds from suitable substrates would not take place (Cardesa, Mirvish, Haven & Shubik, 1974; Mirvish, Gold, Eagen & Arnold, 1974; Sen & Donaldson, 1974).

In the experiments described here, the blocking of the ETU–$NaNO_2$ interaction by the presence of ascorbic acid or ascorbate ion was concluded from the positive teratogenic bioassay that was observed when these chemicals were administered simultaneously (Table 3). This evidence supported the assumption that the formation of *N*-nitrosoethylenethiourea from ETU and $NaNO_2$ was inhibited by the presence of ascorbic acid or ascorbate in the gastro-intestinal tract.

*N*-Nitrosoethylenethiourea has been shown to cause a pattern of CNS anomalies prenatally and hydrocephaly postnatally, both of which were indistinguishable from those induced by ethylenethiourea (Khera & Iverson, 1980). However, to produce a pattern of anomalies somewhat similar to that given by 60 mg ETU/kg, *N*-nitrosoethylenethiourea would be required in a dose of about 120 mg/kg. The maximum hypothetical amount of *N*-nitrosoethylenethiourea that could be derived from a 60-mg/kg dose of ETU (plus 80 mg $NaNO_2$/kg) in the stomach would be equivalent to a sub-teratogenic dose of 77 mg/kg, assuming a 100% yield from the ETU. However, a yield approaching 100% for a nitroso compound has not yet been reported. The maximum yields obtained in the stomach have been 0·2% for diethylnitrosamine (Sen, Smith & Schwinghamer, 1969), 0·5% for nitrosopiperidine (Alam, Saporoschetz & Epstein 1971), 4% for nitrosomethylaniline (Sander, Schweinsberg & Menz, 1968), 8·7 and 27%, respectively, for ethyl- and methylnitrosoureas (Mirvish & Chu, 1973), 15% for nitrosophenmetrazine (Greenblatt, Kommineni & Conrad, 1972) and 30% for diphenylnitrosamine (Sander *et al.* 1968).

A partial to complete loss of ETU's malformative potential on the pre- and postnatal development of rats on combined ETU/$NaNO_2$ treatment was interpreted as an indication of intragastric formation of another teratogen, *N*-nitrosoethylenethiourea, but at a dose probably inadequate to induce teratogenic responses.

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# HEPATIC EFFECTS OF *R*-GOITRIN IN SPRAGUE–DAWLEY RATS

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**Abstract**—In male and female rats, *R*-goitrin treatment increased the relative liver weights; in some of the enlarged livers a high incidence of mitotic figures was observed. *R*-goitrin treatment increased the relative thyroid weight, more in male rats than in females. The thyroids of *R*-goitrin treated rats were either hyperplastic or had most follicles filled with pale-staining colloid and rarely follicles with normal, well-stained colloid. At certain doses, *R*-goitrin increased serum triglycerides, cholesterol, total protein, albumin and calcium, but it decreased serum thyroxine and urea. However, most of these changes in serum chemistry were small. *R*-goitrin caused a temporary increase in urinary ascorbic acid output in both sexes, but the liver ascorbic acid level was increased only in female rats. The duration of pentobarbital-induced sleep was significantly prolonged by *R*-goitrin pretreatment only in male rats.

## INTRODUCTION

The discovery of the goitrogenic effect of cabbage in rabbits (Chesney, Clawson & Webster, 1928; Webster & Chesney, 1930), initiated the search for the responsible substance. In 1949, Astwood, Greer & Ettlinger identified goitrin (L-5-vinyl-2-thiooxazolidone) as one of the active compounds. The natural precursor of goitrin, in cruciferous vegetables, is the glucosinolate progoitrin, isolated by Greer (1956). Other plant goitrogens include allylisothiocyanate and thiocyanate ion (Langer, 1966; VanEtten & Tookey, 1979).

Goitrin has been tested for possible teratogenic action and was found to be non-teratogenic (Khera, 1977; Nishie & Daxenbichler, 1980; Rakalska & Dzierzawski, 1972). Cruciferous vegetables can induce liver and intestinal enzymes (Babish & Stoewsand, 1975; Wattenberg, 1971), can stimulate the metabolism of certain drugs in man and animals (Alvares, Pantuck, Anderson *et al.* 1979; Pantuck, Hsiao, Loub *et al.* 1976; Pantuck, Pantuck, Garland *et al.* 1979) and can inhibit or prevent the hepatic toxicity of polybrominated biphenyls and aflatoxin $B_1$ (Boyd, Sell & Stoewsand, 1979; Stoewsand, Babish & Wimberly, 1978). In addition, chronic feeding of cruciferous vegetables has been shown to increase the weights of certain organs (liver, kidneys, adrenals and thyroid) of the animals. Since cruciferous vegetables are rich in glucosinolates, it is possible that glucosinolates or their degradation products (goitrin, nitriles, isothiocyanates, indoles etc.) are the chemicals responsible for all of the above-mentioned biological effects. Some of the degradation products of glucosinolates containing an indole ring are capable of inducing liver enzymes (Loub, Wattenberg & Davis, 1975).

In a previous publication (Nishie & Daxenbichler, 1980), the increase in relative liver weight of male rats treated with goitrin was reported. The purpose of the present study was to continue the investigation of the effect of *R*-goitrin on the liver in order to permit comparison with the conventional physiological response of the thyroid.

## EXPERIMENTAL

*R-goitrin preparation.* A batch of *Crambe abyssinica* seed which had been in storage for several years was tested and found to have an active thioglycosidase system that converted *epi*-progoitrin to *R*-goitrin with only a trace of nitrile production (VanEtten, Daxenbichler, Peters & Tookey, 1966). The dehulled seed was flaked and then defatted by percolation (room temperature) of pentane-hexane. The flakes were steeped in fresh solvent five times allowing 1–2 hr contact each time. The defatted flakes were spread in a shallow layer and air-dried. Production of goitrin, from the defatted seed flakes, and its subsequent purification was then carried out. One to 1·5 litre water was blended into 0·5 kg defatted flakes and allowed to stand for 10–15 min at room temperature. Dichloromethane (2 litre) was then mixed with the meal slurry and stirred intermittently for 15 min; the whole mix-

*Abbreviations*—PG = Propylene glycol; DMSO = Dimethylsulphoxide; P = Inorganic phosphorus; BUN = Blood urea nitrogen; PAS = Periodic acid-Schiff.

ture was then filtered through a layer of Celite on Whatman No. 1 paper. The residual meal and Celite were again extracted by the same procedure with 2 litre fresh dichloromethane. The combined filtrates were concentrated to a syrup on a rotary evaporator (45°C). The syrup was made alkaline by adding 300 ml 1 N NaOH and then transferred to a separatory funnel. The alkaline solution was shaken with *n*-hexane (300 ml each) and the hexane discarded, then twice with dichloromethane (600 ml each) and the dichloromethane discarded. The alkaline solution was then neutralized by addition of solid $KH_2PO_4$ to pH 7 as determined using pH paper. After addition of the $KH_2PO_4$, goitrin was readily extracted into three 600-ml volumes of dichloromethane. The solvent was removed and *R*-goitrin crystallized readily. Recrystallization from diethyl ether yielded white crystals estimated by UV spectroscopy and GLC at 99–100% purity compared to the reference compound.

*Animal experiments.* Male and female Sprague–Dawley rats weighing 150–200 g were used as test animals; they were maintained on commercially available Teklad* mouse/rat diet (Mogul Corp., Winfield, IA), with 1% iodized salt. PG was used as solvent for *R*-goitrin in all cases, except one in which a 1:1 mixture of PG and DMSO was used. Six to nine control animals from each shipment of 36 rats, received 0·5–1 ml/kg of the solvent sc and the remainder received the same volume of goitrin solution. The test animals received one to four daily doses of goitrin solution (nine rats/dose), and 24 hr after the last dose, the rats were anaesthetized with 30–40 mg/kg of sodium pentobarbital administered ip and blood samples were collected for the following serum chemical analyses; triglyceride, cholesterol, thyroxine, inorganic P, total protein, albumin, calcium, glucose, BUN, alkaline phosphatase, serum glutamic–oxaloacetic transaminase, serum glutamic–pyruvic transaminase, lactate dehydrogenase, creatine phosphokinase, α-hydroxybutyric dehydrogenase. These serum analyses, except thyroxine, were performed at 30°C, using a microcentrifugal analyser (IL Multistat III, Instrumentation Lab., Lexington, MA) and IL reagent kits. The enzyme-immunoassay methodology was used for thyroxine determination, at 37°C, using the Syva EMIT CFA thyroxine assay kit (Syva Co., Palo Alto, CA) and a microcentrifugal analyser. Thyroxine was determined in serum samples stored in cryotubes at −80°C, for 40 to 240 days. After collecting the blood, the following organs were removed, weighed and fixed in buffered formalin for histological studies: liver, kidneys, adrenals and thyroid. PAS and haematoxylin were used as histological stains. In four experiments, the dry weights of livers, from control and *R*-goitrin treated rats were determined by placing 200–300 mg of liver in a vacuum oven set at 29 lb vacuum and 65°C for 3 days.

*Ascorbic acid determination.* In some experiments, the ascorbic acid concentration in the liver, adrenals and urine was determined by the method of Schaffert & Kingsley (1955). The adrenal glands and about 1 g of liver were weighed and placed in cold 6% trichloroacetic acid solution, minced and stored at −80°C, for later analysis of ascorbic acid. The effect of *R*-goitrin on daily output of urine and urinary ascorbic acid and water intake was measured daily by placing nine male and nine female rats in clean metabolism cages supplied with food, water and urine collecting cups containing 5 ml of 10% oxalic acid (for ascorbic acid analysis). The average control values in the output of urine, urinary ascorbic acid, and water intake was obtained from data gathered on two nonconsecutive days preceding the *R*-goitrin injection. Subsequently, 50 mg *R*-goitrin/kg/day was injected sc for 2 days, and the same parameters were measured, starting on the days of injection and on predetermined days. Single higher doses of *R*-goitrin were also tested and the results were compared with the corresponding pretreatment values and analysed by Student's paired *t*-test. In these cases, the urine was collected and analysed for 1 day only. In two cases, naïve control rats of the same batch and age were tested alongside *R*-goitrin treated rats and the data was analysed by Student's non-paired *t* test.

*Enzyme-induction tests.* The possibility of enzyme-induction by *R*-goitrin was tested indirectly by measuring the duration of sleeping time induced by ip injection of either 120 mg hexobarbital/kg or 30–40 mg sodium pentobarbital/kg, 24 hr after the last dose of *R*-goitrin. The duration of sleeping time was compared with that of the control group pretreated with the solvent, propylene glycol.

*Statistical analysis.* The changes caused by *R*-goitrin in serum chemistry, relative organ weights, liver dry weights, liver ascorbic acid, and duration of sleeping time were analysed by Student's non-paired *t* test. These changes were expressed as a percentage change by comparison with mean control values from rats of the same age and shipment, treated only with solvent (PG or PG/DMSO).

## RESULTS

### *Serum chemistry*

The serum chemistry values for control rats are presented in Table 1. Female controls had lower serum values than did males in all but two parameters, namely, albumin and cholesterol. The changes in serum chemistry caused by the different doses of *R*-goitrin are summarized in Table 2. *R*-goitrin increased serum triglyceride and cholesterol and decreased thyroxine at some doses. The decrease in serum thyroxine was found at more dose levels in male rats than in females treated with *R*-goitrin. Excluding the group treated with *R*-goitrin dissolved in DMSO/PG, the size of the increase in serum cholesterol in male rats increased with the total dose of *R*-goitrin administered. At certain doses, *R*-goitrin increased total protein, albumin and calcium and decreased BUN but these changes were not dose-related. Changes in serum glucose and P also were not dose-related and they may be due to animal variability or other causes.

Serum enzymes were not significantly changed in rats treated with *R*-goitrin dissolved in PG, whereas serum from rats which received *R*-goitrin dissolved in DMSO/PG had increased lactate dehydrogenase and α-hydroxybutyric dehydrogenase and decreased alka-

*The mention of this product does not imply recommendation over similar products that may be available.

Table 1. *Control values of serum chemistry, relative organ weights, liver dry weights, liver ascorbic acid and adrenal ascorbic acid of Sprague–Dawley rats of the same age as those treated*

| Parameter | Male (mean ± SEM)† | Female (mean ± SEM)† | Difference between sexes (%) |
|---|---|---|---|
| Body weight (g) | 215 ± 3·56 (71) | 179 ± 2·31 (54) | −17** |
| Serum chemistry | | | |
| Triglyceride (mg/dl) | 70 ± 2·49 (71) | 54·2 ± 2·61 (54) | −22** |
| Cholesterol (mg/dl) | 79 ± 1·59 (70) | 90 ± 1·7 (54) | +13·8** |
| Thyroxine (μg/dl) | 4·2 ± 0·068 (60) | 3·97 ± 0·06 (54) | −5·4* |
| Albumin (g/dl) | 3·38 ± 0·026 (71) | 3·57 ± 0·03 (54) | +4·7** |
| BUN (mg/dl) | 25·3 ± 0·5 (71) | 24·3 ± 0·485 (54) | NS |
| Total protein (g/dl) | 5·91 ± 0·116 (71) | 5·9 ± 0·076 (54) | NS |
| Calcium (mg/dl) | 10·4 ± 0·092 (71) | 10·1 ± 0·102 (54) | −3* |
| Inorganic P (mg/dl) | 10·2 ± 0·131 (70) | 8·5 ± 0·179 (54) | −17** |
| Glucose (mg/dl) | 142 ± 2·02 (71) | 132 ± 2·12 (54) | −7** |
| Relative organ weights | | | |
| Liver (g/kg) | 47·6 ± 0·507 (63) | 39·4 ± 0·128 (63) | −17** |
| Thyroid (mg/kg) | 69·8 ± 1·6 (63) | 75·4 ± 1·726 (63) | +8·05* |
| Adrenals (mg/kg) | 193 ± 2·81 (63) | 314 ± 4·22 (62) | +62·7** |
| Kidneys (g/kg) | 8·91 ± 0·087 (63) | 8·17 ± 0·095 (63) | −8·3** |
| Liver dry weight (%)‡ | 31·07 ± 0·125 (35) | 30·34 ± 0·11 (36) | −2·3** |
| Liver ascorbic acid (μg/g) | 312 ± 9·85 (36) | 244 ± 9 (36) | −22** |
| Adrenal ascorbic acid (μg/mg) | 4·7 ± 0·132 (36) | 3·79 ± 0·127 (35) | −19·4** |

NS = Not statistically significant

†The numbers of rats examined are given in parenthesis.

‡The liver dry weights are given as a percentage of the wet weight of the liver.

Values marked with asterisks indicate a statistically significant difference (Student's non-paired *t* test) between the values for the two sexes (*$P < 0.05$; **$P < 0.01$).

line phosphatase, compared with corresponding controls treated only with DMSO/PG.

*Relative organ weight changes*

In the controls, the relative liver and kidney weights were lower in female than in male rats, but the relative adrenal and thyroid weights were higher than in males (Table 1). *R*-goitrin increased the relative liver weights in both sexes, at all of the doses tested (Table 2). In male rats, the greatest increase in relative liver weight (+45%) was produced by a total dose of 160 mg/kg (40 mg/kg/day for 4 days); some livers in this group had a high incidence of mitotic figures (Fig. 1c). In female rats, the greatest increase in liver weight (+33%) was produced by a dose of 200 mg/kg (100 mg/kg/day for 2 days). In most of the experimental groups (6/8), *R*-goitrin had no effect on the dry weights of livers expressed as a percentage of wet weight. The relative kidney weights were not changed by *R*-goitrin, even at a total dose of 200 mg/kg. At certain doses, *R*-goitrin affected the adrenal weights in males, but had no effect on adrenals from females. The relative thyroid weights were increased in most experimental groups. This increase was found at more dose levels in males than in females.

*Liver and adrenal ascorbic acid*

In female rats, *R*-goitrin increased the liver ascorbic acid (Table 2), but in males no significant changes were seen at three of four dose levels. In most experimental groups adrenal ascorbic acid was not significantly affected.

*Urine volume, urinary ascorbic acid and water intake*

*R*-goitrin increased the urinary ascorbic acid output in both sexes, at all doses tested, when compared with the pretreatment level of ascorbic acid, or the ascorbic acid output of naïve control rats placed in the metabolism cage for the first time (Table 3). However, the increased ascorbic acid output with two doses of 50 mg/kg sc of *R*-goitrin did not persist beyond 1 day (male rats) or 6 days (female rats), after the cessation of *R*-goitrin treatment. The ascorbic acid output was considerably higher in male control rats than in female controls, in most cases, as can be seen in the pretreatment levels of ascorbic acid output and in naïve controls rats (Table 3).

In female rats, *R*-goitrin increased the 24-hr urinary output and the water intake at all doses tested (Table 3), while in male rats, neither urinary output nor water intake was significantly changed. The water intake and urinary output of male and female control rats were not significantly different.

*Hexobarbital or sodium pentobarbital sleeping times*

Male rats pretreated with 30 or 40 mg *R*-goitrin/kg/day for 4 days showed significantly increased pentobarbital-induced (40 mg/kg ip) sleeping time (Table 4). The sleep induced by 120 mg/kg ip hexobarbital or 30 mg/kg ip of pentobarbital was not prolonged by *R*-goitrin pretreatment in males or females. The duration of sleep in female control rats was significantly longer than in male controls, namely 3·29 times with 120 mg/kg ip hexobarbital, and 2·4 times with 30 mg/kg ip sodium pentobarbital.

*Histological changes caused by R-goitrin and the solvent DMSO/PG*

Preliminary histological studies showed an unusual number of mitotic figures in some livers of rats treated with *R*-goitrin dissolved in either PG (Fig. 1c)

or DMSO/PG (Fig. 1e). Control rats treated with the solvent mixture DMSO/PG also had some mitotic figures in the liver, whereas other control rats, treated with PG (Fig. 1a) or untreated, showed rare or no mitosis. The hepatocytes in different stages of mitosis were not stained by PAS and were lighter than surrounding hepatocytes with glycogen. In general, goitrin-treated rat livers had glycogen comparable with that of the corresponding controls, but some goitrin-treated rat liver had one or two layers of hepatocytes surrounding the periportal area without glycogen.

In control thyroid sections the follicles were lined with flat epithelial cells and filled with uniformly well-stained colloid (Fig. 1b). *R*-goitrin treated rat thyroid had most follicles filled with pale-staining colloid and rarely, follicles with well-stained colloid (Fig. 1d). Other thyroids of *R*-goitrin treated rats were hyperplastic, had small follicles with scanty and pale-staining colloid, and had rare follicles with well-stained colloid (Fig. 1f).

Rats treated with sc DMSO/PG, or *R*-goitrin dissolved in DMSO/PG, showed kidney and thyroid lesions. The walls of the urine-collecting tubules in the medullary zone of the kidneys were partly destroyed by DMSO/PG and an interconnecting lumen was formed with the adjacent tubules. This newly formed lumen was filled with cellular debris or pink-staining material. These kidney lesions were not extensive. The rats in this group had haematuria within 1 hr after sc injection, while rats treated with PG or *R*-goitrin dissolved in PG had neither haematuria nor kidney histological lesions. The thyroid lesions, caused by DMSO/PG or *R*-goitrin dissolved in DMSO/PG, consisted of necrosis of epithelial cells lining the

Table 2. *Effects of* R-*goitrin on serum chemistry, relative organ weights, liver dry weights, and liver and adrenal ascorbic acid levels*

| Parameter | *R*-goitrin dose (mg/kg × days)... Solvent... | Percentage change compared with corresponding mean control value | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 30 × 4 PG | 40 × 3 PG | 40 × 4 PG | 50 × 2 PG | 50 × 4 PG | 100 × 2 PG | 100 × 2 PG-DMSO |
| **Males** | | | | | | | | |
| Serum chemistry | | | | | | | | |
| Triglyceride | | NS | NS | +27* | +47* | NS | +44* | +53* |
| Cholesterol | | +17** | +22** | +28** | NS | +31** | +28** | +10·7* |
| Thyroxine | | −26** | −23** | −21** | —† | −11** | NS | −17** |
| Albumin | | NS | NS | +14·6** | NS | +5·3* | NS | +6·7** |
| BUN | | NS | −19** | NS | −17** | NS | NS | −11** |
| Total protein | | NS | +14** | NS | NS | NS | +9·8** | NS |
| Calcium | | +8·5** | NS | NS | +11·9** | NS | NS | +12* |
| Inorganic P | | NS | −13** | NS | NS | −12·2** | NS | NS |
| Glucose | | NS | −6·8* | NS | NS | NS | −8* | −10** |
| Change in relative organ weights (%) | | | | | | | | |
| Liver | | +27* | +29** | +45** | +11·5** | +27** | +24** | +12** |
| Thyroid | | +30** | NS | +26** | +12** | +22** | NS | +11* |
| Adrenals | | NS | NS | −11* | +19·7* | NS | NS | +9·7** |
| Kidneys | | NS | NS | NS | NS | NS | NS | NS |
| Liver dry weight | | | | | NS | +4·2** | NS | NS |
| Liver ascorbic acid | | | | | NS | NS | +12* | NS |
| Adrenal ascorbic acid | | | | | −8·2* | NS | NS | NS |
| **Females** | | | | | | | | |
| Serum chemistry | | | | | | | | |
| Triglyceride | | NS | +51** | +107* | +37* | NS | +77** | NS |
| Cholesterol | | +22** | +17** | +13·8** | NS | +12·1* | NS | +24** |
| Thyroxine | | −12** | NS | NS | −18** | NS | NS | —† |
| Albumin | | +13·7** | +4·8* | +13·8* | NS | NS | +8* | NS |
| BUN | | −17** | NS | NS | −11* | NS | NS | −20** |
| Total protein | | +12** | NS | NS | NS | +8·3* | +5·8* | NS |
| Calcium | | +7·1* | NS | +12·7** | NS | NS | NS | NS |
| Inorganic P | | NS | −13* | +7·7** | +11·4* | NS | −16** | +33** |
| Glucose | | NS | NS | NS | NS | NS | +9·9* | −7* |
| Change in relative organ weights (%) | | | | | | | | |
| Liver | | +20** | +25** | +23** | +29** | +13·5** | +33** | +14·5** |
| Thyroid | | +25** | NS | +24** | NS | NS | NS | NS |
| Adrenals | | NS | NS | NS | NS | NS | NS | NS |
| Kidneys | | NS | NS | NS | NS | NS | NS | NS |
| Liver dry weight | | | | | NS | NS | +4·6* | NS |
| Liver ascorbic acid | | | | | +19·3* | +18* | +17** | +14** |
| Adrenal ascorbic acid | | | | | NS | NS | +27** | NS |

PG = Propylene glycol DMSO = Dimethylsulphoxide NS = Not statistically significant

†Data not available.

Values marked with asterisks showed a significant difference (Student's non-paired *t* test, 16 degrees of freedom) between the experimental group (nine rats) and the corresponding control group (nine rats) (*$P < 0.05$; **$P < 0.01$).

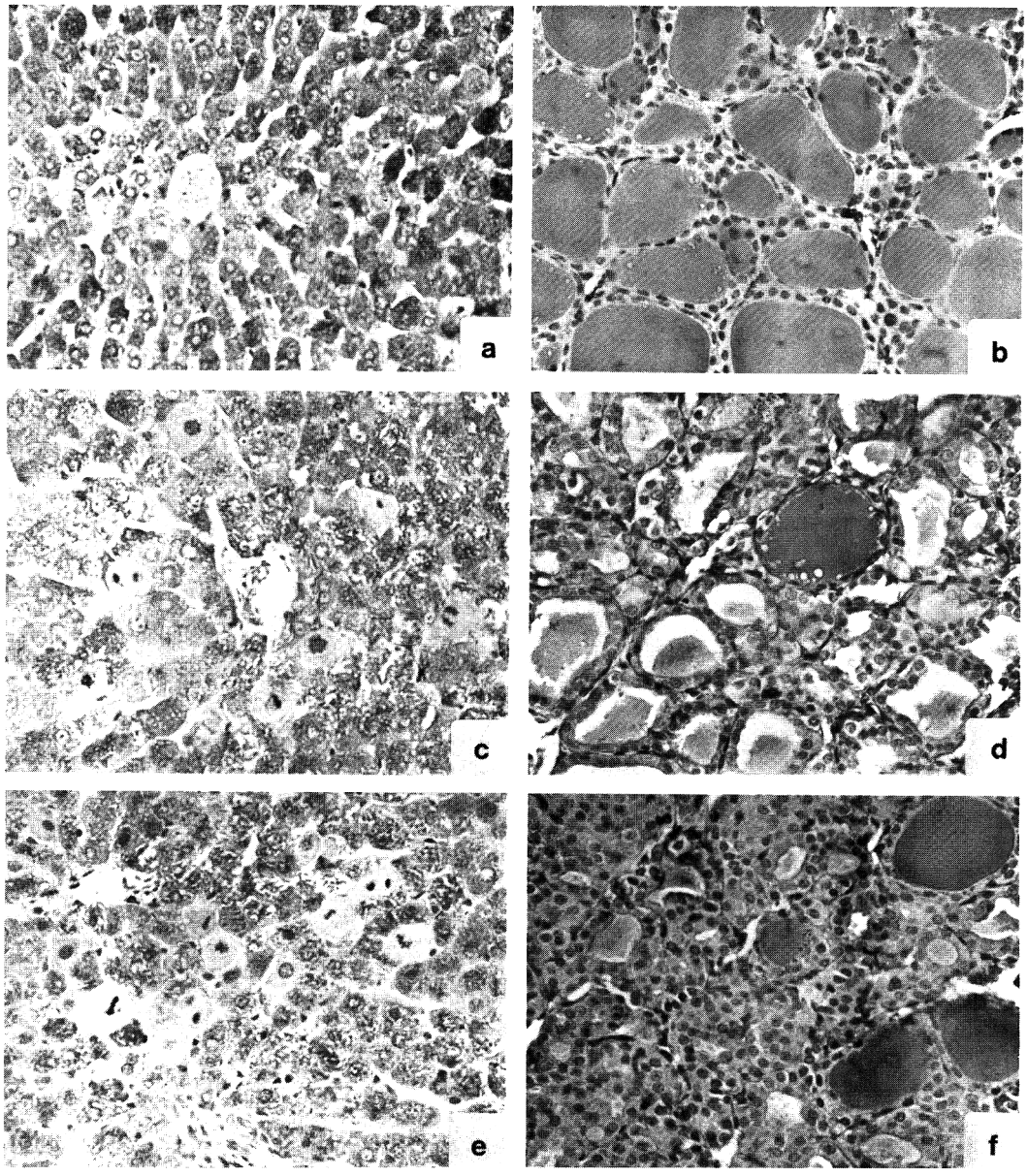


Fig. 1. Liver and thyroid sections stained with periodic acid-Schiff (PAS) and haematoxylin, ×300: (a) male control liver; (b) male control thyroid; (c) liver of male rat treated with *R*-goitrin (40 mg/kg/day sc for 4 days) and killed 24 hr after the last dose showing the numerous mitotic figures surrounding the centrolobular vein; (d) thyroid section of male rat treated with *R*-goitrin (40 mg/kg × 4 days), showing that most thyroid follicles have pale-staining colloid, only one has well-stained normal colloid; (e) liver section of a male rat treated with *R*-goitrin (100 mg/kg × 2 days, dissolved in DMSO/PG) showing numerous mitotic figures near the periportal area; (f) thyroid section of a male rat treated with *R*-goitrin (50 mg/kg × 4 days), showing marked hyperplasia and only three follicles with well-stained colloid. (When not specified, *R*-goitrin was dissolved in propylene glycol.)

Table 3. *Effects of* R-*goitrin on urinary ascorbic acid, urine output and water intake in rats*

| Age (days) | *R*-goitrin dose and route | No. of rats in group | Male | | | Female | | |
|---|---|---|---|---|---|---|---|---|
| | | | AA output (mg/kg/day or % change) | Urine output (ml/kg/day or % change) | Water intake (ml/kg/day or % change) | AA output (mg/kg/day or % change) | Urine output (ml/kg/day or % change) | Water intake (ml/kg/day or % change) |
| | | | **Naïve controls†** | | | | | |
| 76 | 0 | 6 | 15·12 ± 2·81 | 49 ± 3·59 | 122 ± 16·3 | 13·4 ± 0·79 | 44 ± 3·55 | 122 ± 6·94 |
| 55 | 0 | 6 | 21·97 ± 2·2 | 67 ± 5·71 | 114 ± 12·25 | 11·7 ± 1·27 | 80·5 ± 10·6 | 135 ± 9·8 |
| | | | **Experimental group 1** | | | | | |
| 52–58 | Pretreatment | 9 | 17·8 ± 1·33 | 51 ± 2·57 | 141 ± 12·33 | 13·54 ± 1·33 | 44 ± 3·8 | 128 ± 11 |
| 62 | 50, sc | | +86%** | +23% | −9·4% | +65%** | +156%** | +60%** |
| 63 | 50, sc | | +79%** | −2·2% | −24% | +91%** | +100%** | +43%** |
| 64 | — | | +18·8%** | +15% | +12% | +75%** | +57%* | +28%** |
| 66 | — | | −11% | +7·5% | −4·2% | +52%** | +18·5% | +18%* |
| 69 | — | | −3·5% | +18% | −10% | +27%* | +45% | +13% |
| 72 | — | | −19% | +30% | −12% | +10·4% | +16% | +11% |
| | | | **Experimental group 2** | | | | | |
| 54 | Pretreatment | 6 | 22·6 ± 2·98 | 58 ± 4·49 | 133 ± 11·84 | 12·91 ± 1·5 | 53 ± 6·047 | 128 ± 13·60 |
| 76 | 100, ip | | +41%** | +19·6% | −22% | +80%** | +158%** | +54%* |
| | | | **Experimental group 3** | | | | | |
| 50 | Pretreatment | 6 | 16·67 ± 1·26 | 71 ± 4·89 | 130 ± 11·84 | 14·21 ± 0·84 | 73 ± 9·4 | 150 ± 15·52 |
| 55 | 200, sc | | +144%** | +40% | −9·1% | +62%** | +131%** | +54%** |
| 56 | — | | +53% | +26% | +8% | +71%** | +108%** | +42%** |

AA = Ascorbic acid

†Measurements for the naïve control groups were made concurrently with measurements for groups 2 & 3 respectively (treatment day) using animals of the same age as those in the corresponding experimental group.

Values for the control groups are means ± SEM. Values for the treated groups are percentage increases or decreases compared with the corresponding pretreatment control values; those marked with asterisks showed a significant difference (Student's paired *t* test) from the corresponding controls (*$P < 0.05$; **$P < 0.01$).

Table 4. *Effect of* R-*goitrin pretreatment on sleeping time induced by hexobarbital or sodium pentobarbital (tested 24 hr after the last dose of* R-*goitrin)*

| | | Males | | | Females | | |
|---|---|---|---|---|---|---|---|
| | | | Sleeping time | | | Sleeping time | |
| *R*-goitrin sc (mg/kg × days) | Barbiturate and ip dose (mg/kg) | No.of rats | Mean ± SEM (min) | % change† | No.of rats | Mean ± SEM (min) | % change† |
| 0 | Hexobarbital (120) | 5 | 23·4 ± 1·88 | | 6 | 77 ± 4·16 | |
| 100 × 2 | Hexobarbital (120) | 6 | 26·5 ± 1·28 | +13·2 | 6 | 85·7 ± 7·35 | +11·2 |
| 0 | Pentobarbital (40) | 9 | 62·11 ± 4·036 | | | | |
| 30 × 4 | Pentobarbital (40) | 9 | 95·44 ± 4·9 | +54** | | | |
| 0 | Pentobarbital (30) | | | | 9 | 117 ± 4·11 | |
| 30 × 4 | Pentobarbital (30) | | | | 9 | 122 ± 11·11 | +4·65 |
| 0 | Pentobarbital (40) | 9 | 70·22 ± 4·87 | | | | |
| 40 × 4 | Pentobarbital (40) | 9 | 94·22 ± 2·63 | +34** | | | |
| 0 | Pentobarbital (30) | | | | 9 | 114 ± 7·8 | |
| 40 × 4 | Pentobarbital (30) | | | | 9 | 101 ± 10·5 | −12 |
| 0 | Pentobarbital (30) | 5 | 48 ± 4·11 | | 6 | 115·5 ± 5·75 | |
| 50 × 4 | Pentobarbital (30) | 6 | 52·33 ± 4·8 | +9 | 7 | 108·43 ± 4·72 | −6·2 |

†Percentage change in sleeping time as a result of *R*-goitrin pretreatment. Values marked with asterisks show a significant increase (Student's non-paired *t* test) compared with the corresponding control value (**$P < 0.01$).

follicles and formation of interconnecting follicles filled with cellular debris. These lesions were not extensive and were not seen in all thyroids.

## DISCUSSION

The increase in serum cholesterol and triglycerides and decrease in thyroxine by *R*-goitrin were expected, since a hypothyroid condition is generally associated with high serum cholesterol. According to Kritchevsky (1960), thyroxine appears to stimulate cholesterol degradation, and therefore *R*-goitrin, by decreasing thyroxine, also decreased cholesterol degradation. Goitrin is well known for its goitrogenic action characterized by the hyperplasia of the thyroid (Fig. 1f), but it also increased the liver weight of male rats (Nishie & Daxenbichler, 1980). In the present study, multiple small doses increased the liver weight in animals of both sexes (Table 2). The increase in liver size and urinary ascorbic acid output produced by chemicals are often indications of liver enzyme induction, with increased drug metabolism. When rats pretreated with *R*-goitrin were tested for possible increases in drug metabolism (shortening of hexobarbital or pentobarbital-induced sleeping time), it was found that male rats pretreated with *R*-goitrin had prolonged sleeping time induced by pentobarbital, while sleeping time remained unchanged in female rats. However, the urinary ascorbic acid was increased by *R*-goitrin in both sexes, but not beyond 6 days after injection (Table 3: Group 1). This increased abscorbic acid output could be caused by increased excretion by the kidneys and depletion of existing ascorbic acid, by increased synthesis of ascorbic acid, or by increased output of some substance acting as an artefact in the urine analyses for ascorbic acid. The liver ascorbic acid content was increased in goitrin-treated female rats, but mostly unchanged in male rats (Table 2). The adrenal ascorbic acid was not changed in most experiments.

The possibility of liver oedema accounting for the increased liver weight can be excluded because liver dry weights were not changed significantly. *R*-goitrin seems to enlarge the liver by direct action, rather than through the thyroid, since the livers were enlarged in all experiments, while thyroid weights and serum thyroxine were changed only in some experiments.

*R*-goitrin seems to belong to the group compounds, including thiourea and thyroxine, reviewed by Barka & Popper (1967), that are capable of enlarging the liver at certain dose levels, without producing obvious microscopic changes. The lack of obvious hepatotoxic changes by *R*-goitrin was confirmed by the normal serum enzyme levels. The increase in the liver weight could be explained by the increased mitosis (Figs 1c & e), resulting in an increase in the total number of hepatocytes, i.e., hyperplasia of the liver caused by *R*-goitrin. The mitogenic effect of *R*-goitrin should be confirmed by using colchicine to determine the mitotic index at fixed time intervals after *R*-goitrin injection. Thiourea, a known goitrogen, also increased mitosis of hepatocytes (Rachmilewitz, Rosin & Doljanski, 1947 & 1950), did not change the liver dry weights and increased urinary output (Doljanski, Eshkol, Givol *et al.* 1955), as was also observed in *R*-goitrin-treated female rats. Thioacetamide is another goitrogenic compound with mitogenic effects on the liver (Reddy, Chiga & Svoboda, 1969). Many antithyroid and related compounds such as thiourea and thioacetamide, with mitogenic properties are known to be carcinogens (Heuper & Conway, 1964; Veljkovic, 1980), therefore, if the mitogenic property of *R*-goitrin is confirmed, its potential carcinogenicity should be investigated. It is interesting that triiodothyronine (T3), which has pharmacologically opposite effects to *R*-goitrin, also has mitogenic effects on the liver (Lee, Sun & Miller, 1968; Short, Wedmore, Kibert & Zemel, 1980).

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# PATULIN MYCOTOXICOSIS IN THE RAT: TOXICOLOGY, PATHOLOGY AND CLINICAL PATHOLOGY

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**Abstract**—Patulin, a secondary metabolite produced by species of the genera Penicillium and Aspergillus, was administered to male Sprague–Dawley rats, weighing 50–60 g, by the oral, sc and ip routes. The 72-hr $LD_{50}$ values (in mg/kg weight) were: oral, 55·0; sc, 11·0; ip, 10·0. Mortality was greatest 0–24 hr after administration by the oral and sc routes and 49–72 hr after ip dosing. Gross alterations consisted of gastric and intestinal hyperaemia and distention. Histopathological alterations consisted principally of ulceration and inflammation of the stomach. Patulin was administered orally to rats daily or every other day for 2 wk at doses of 50 or 75% of the oral $LD_{50}$. Mortality in the treated groups was greater than in controls but was similar for all treated groups. No evidence of cumulative toxicity was found and the gross and histopathological alterations were similar to those found in the $LD_{50}$ studies. Clinicopathological alterations included metabolic alkalosis with respiratory compensation, oliguria, decreased serum sodium, elevated blood glucose, reduced plasma protein and an elevated total leucocyte count which differential leucocyte counts indicated to be due to neutrophilia. The inflammatory alterations observed in the gastro-intestinal tract may be due to the irritant properties of patulin or to an alteration in the gastro-intestinal flora by the antibiotic activity of patulin.

## INTRODUCTION

Patulin is a secondary metabolite of a number of fungal species belonging to the genera Penicillium and Aspergillus (Abraham & Florey, 1949). Patulin has been most commonly isolated from apples and apple products contaminated with the common storage-rot fungus of apples, *P. expansum* (Brian, Elson & Lowe 1956; Harwig, Chen, Kennedy & Scott, 1973; Wilson & Nouvo, 1973). In addition, patulin has been obtained from rotten fruits (Buchanan, Sommer, Fortlage *et al.* 1974; Frank, 1977), mouldy feeds (Wilson, 1976) and stored cheese (Bullerman, 1976).

Patulin was reported to be carcinogenic in rats when administered sc (Dickens & Jones, 1961). However, it was not carcinogenic in rats when administered orally for 64 wk (Osswald, Frank, Komitowski & Winter, 1978). It is also a potential teratogenic and mutagenic agent since it has been found to cause mitotic spindle alteration accompanied by chromosome fragmentation in segmenting salamander eggs (Sentein, 1955) and to inhibit mitosis in chick-embryo cultures (Ciegler, Vesonder & Jackson, 1977).

The acute toxicity of patulin for rats has been studied by several authors, and the results of different investigators have varied. Escoula, More & Baradat (1977) reported congestion and haemorrhages in multiple organs of adult rats, gastric and jejunal ulcers and renal and splenic necrosis. Pleural and peritoneal exudates and pulmonary oedema were also found in treated rats. Katzman, Hays, Cain *et al.* (1944) reported pulmonary oedema, visceral congestion and renal tubular degeneration.

The toxicological responses obtained in subacute studies have also varied. Dailey, Brouwer, Blaschka *et al.* (1977) reported few lesions in patulin-treated rats and gaseous distention of the gastro-intestinal tract was the only alteration common in test rats. Hepatic cell necrosis, pulmonary atelectasis, alveolar septal congestion and alveolar haemorrhages have also been reported (Hayes, Phillips, Williams & Ciegler, 1979).

Clinical pathological data obtained following patulin administration to rats are limited. Katzman *et al.* (1944) and Broom, Bülbring, Chapman *et al.* (1944) observed that urine output was reduced markedly following sc patulin administration. Haemoglobulin and packed cell volume values increased markedly when patulin was administered orally at 20 mg/kg body weight (Broom *et al.* 1944). Dailey *et al.* (1977) determined clinical chemistry and haematological values in rats administered patulin orally at 1·5 mg/kg body weight, 5 days/wk for 23 wk. No significant alterations were found.

The mechanism of patulin toxicity is unknown. The antibiotic properties of patulin on intestinal microflora were proposed as contributory for certain of the toxicological effects (Dailey *et al.* 1977; McKinley & Carlton, 1980a,b). Patulin inhibited the aerobic respiration of bacteria (Gottlieb & Singh, 1964) and phagocytic cells (Delannay, Daniel, De Roquefeuil & Henon, 1955), but there are no data to suggest that this is an effect of the mycotoxin *in vivo*.

Following oral administration of [$^{14}$C]patulin to rats, 70% was recovered in a metabolized form from the urine and faeces in 24 hr. The major retention site for $^{14}$C-activity was the erythrocyte. Again, such data do not provide a basis for explaining the toxicity of patulin.

The present studies were undertaken to define the effects of acute patulin mycotoxicosis in rats following oral, sc and ip administration, to determine the effects of multiple oral doses of patulin and to determine the clinicopathological alterations in rats following oral patulin administration.

## EXPERIMENTAL

*Animals.* Male Sprague–Dawley rats were purchased from Harlan Industries, Cumberland, IN. Rats for trials I–III weighed 40–60 g and were randomly assigned to clear plastics cages with ground corn cobs (Bed-o-Cobs, American Cob Mills, Anderson, IN) as bedding material. Water and feed (Wayne Laboratory Animal Diet, Allied Mills, Castleton, IN) were supplied *ad lib.* Rats for trials IV and V weighed 175–200 g and were housed individually in wire-bottomed metabolism cages during the experiments. The ambient temperature was about 22°C and the relative humidity was 55 ± 5%. Rats were maintained under natural lighting with approximately 16-hr daylight and 8-hr darkness. They were transferred to clean cages with fresh bedding twice weekly and were acclimatized to their surroundings for 3 days before dosing.

*Collection of tissues.* Rats found dead during the experiment and survivors killed by cervical dislocation at termination were necropsied. Multiple tissues were immersion fixed in neutral buffered 10% formalin. The lungs and distal trachea were removed intact, infused with formalin to 12 cm of water pressure, tied with string to prevent reflux and immersed in the fixative. Fixed tissues were dehydrated, embedded in paraffin, sectioned and stained with haemotoxylin and eosin for histopathological examination.

*Materials.* Purified patulin (lot number U-4847, Upjohn Company, Kalamazoo, MI) was dissolved in 0·1 mM-citrate buffer (pH 5·0) to give concentrations of 4·7, 1·1 and 7·5 mg/ml for oral, ip and sc dosing, respectively. For the multiple-dose experiment, patulin was mixed at a concentration of 5·5 mg/ml buffer every other day and for the single-dose, 14-day experiment it was mixed at 7·0 mg/ml. For the clinical pathology experiments patulin was mixed at a concentration of 14·7 mg/ml. The rats were weighed immediately before dosing. For the initial dosing, feed was withheld for 12 hr before administration of the toxin or control solvent. Oral dosing was by gastric intubation using an 18-gauge, 1·5-in. animal-dosing needle. A tuberculin syringe with a 25-gauge needle was used for the sc and ip injections.

### *Design of experiments*

*Trial I.* The single-dose toxicity of patulin was evaluated in groups of ten rats after oral, sc and ip administration. Control animals were given similar amounts of the solvent. The $LD_{50}$ values and 95% confidence intervals were calculated (Litchfield & Wilcoxon, 1949).

*Trial II.* Multiple doses of patulin of 50 and 75% of the oral $LD_{50}$ (28 and 41 mg/kg body weight, respectively) were given either every day or every other day for 1 wk. The doses given during the second week remained the same or were increased from 28 to 41 mg/kg but the frequency of dosing remained the same. Ten animals were randomly assigned to each group and one control group was given the solvent at a volume equivalent to the greatest quantity used for test groups.

*Trial III.* To determine the subacute effects of a single oral dose of patulin, two groups of 20 rats each were administered a single oral dose of patulin at 50 and 75% of the oral $LD_{50}$ (28 and 41 mg/kg body weight, respectively). The control group of 20 rats was given the solvent at a volume equivalent to the greatest quantity used for test groups. Animals were killed 14 days after dosing.

*Trial IV.* Certain clinicopathological tests were carried out after the induction of patulin mycotoxicosis. Test rats were given a single dose of 41 mg patulin/kg body weight (75% of the oral $LD_{50}$) and control rats were given an equivalent volume of 0·1 M-citrate buffer. Both groups were placed in wire-bottomed metabolism cages in which water and feed were supplied *ad lib.* Groups of rats were killed for collection of blood and urine at 0, 3, 12 and 24 hr after treatment. Sufficient rats were included in each group for ten samples for each determination to be available.

Urine samples collected at the above intervals were observed for colour and clarity. Specific gravity was determined using a refractometer (TS meter, American Optical, Keene, NH). The pH of the urine and tests for protein, glucose, ketones, bilirubin, urobilinogen and blood were done with diagnostic reagent strips (Multistix, Ames Co., Elkhart, IN).

Blood was collected from the right ventricle following opening of the thorax under ether anaesthesia and samples were placed in a Micro Blood Collecting Tube (catalogue No. 477376, Corning Medical and Scientific Co., Medfield, MA) containing potassium EDTA for blood gas determinations, in a glass collection tube with potassium EDTA (Vacutainer, Becton-Dickinson & Co., Rutherford, NJ) for haematology or in tubes without anticoagulant for chemical determinations.

Total erythrocyte and leucocyte counts were determined by an electronic counting device (Coulter Counter, Model Fn, Coulter Electronics, Inc., Hialeah, FL). Haemoglobin concentrations were determined by a cyanmethaemoglobin method (Coulter Haemoglobinometer, Coulter Electronics, Inc.). Packed cell volume was determined by a standard microhaematocrit method and total plasma protein was determined using a refractometer (TS meter, American Optical). Blood smears were stained with Wright's stain using an Ames Hema-Tak slide stainer (Ames Co., Elkhart, IN).

After centrifugation, serum was taken for chemical determinations. Sodium and potassium concentrations were determined by flame photometry (Flame Photometer, model 143, Instrumentation Laboratories, Boston, MA) and chloride concentrations were measured by coulometry (Corning 920 chloridometer).

Blood urea nitrogen determinations were made by an enzymatic conductivity rate method (Beckman BUN Analyzer 2, Beckman Instruments, Palo Alto, CA). Measurement of $O_2$ was made using a Clark-type electrode. A Severinghaus-Stow-type electrode was used to measure $CO_2$ and a flow-through glass capillary and reference assembly was used to determine pH (Corning pH/Blood Gas 165 Analyzer). This apparatus provided determinations of $CO_2$, $HCO_3$ and base excess on the basis of the Henderson–Hasselbalch equation.

Blood glucose was determined by the hexokinase method (Worthington Statzyme, Catalogue no. 27536, Worthington Diagnostics Inc., Freehold, NJ) and a

Table 1. *Number of rats dying and time to death after patulin administration (trial I)*

| Route of administration | Patulin dose (mg/kg body weight) | Time after dosing (hr)... | No. of deaths* 0–24 | 25–48 | 49–72 |
|---|---|---|---|---|---|
| Oral | 0 | | 0 | 0 | 0 |
| | 50 | | 4 | 0 | 0 |
| | 55 | | 3 | 0 | 0 |
| | 60 | | 4 | 0 | 0 |
| | 65 | | 6 | 0 | 0 |
| Subcutaneous | 0 | | 0 | 0 | 0 |
| | 8 | | 3 | 0 | 0 |
| | 10 | | 5 | 0 | 0 |
| | 12 | | 10 | 0 | 0 |
| Intraperitoneal | 0 | | 0 | 0 | 0 |
| | 9 | | 0 | 0 | 3 |
| | 11 | | 0 | 0 | 7 |
| | 13 | | 4 | 2 | 0 |

*There were ten animals in each group.

dual-beam recording spectrophotometer (Beckman Model 25 Spectrophotometer 15). Alanine aminotransferase determinations were made kinetically (Biodynamics/BMC GPT optimized kit, Biodynamics/BUC Inc., Indianapolis, IN).

*Trial V.* To evaluate alterations in blood gases, sodium, potassium and chloride over the period of greatest change (as determined in trial IV), rats were given a single oral dose of 55 mg patulin/kg body weight. Control rats were given an equivalent volume of solvent. Blood was collected 0, 45, 90, 180 and 360 min after treatment and tested by methods identical to those used in trial IV.

*Statistical analysis.* Statistical analysis of clinicopathological data was completed, using the program *Statistical Package for the Social Sciences* (Nie, 1980) at the Purdue University computer center. Homogeneity of variances was determined by the Cochran and the Bartlett–Box tests. Normality of data was determined by the Shapiro–Wilks test. Data were analysed using a two-way analysis of variance and the Student–Newman–Keuls procedure. Differences between observed means were considered statistically significant when $P \leqslant 0{\cdot}05$.

## RESULTS

### *Trial I*

In this study of the acute toxicity of a single dose of patulin, clinical signs were similar regardless of the route of administration. Control and treated rats showed signs of agitation and irritation manifested by rapid movement around the cages. Excessive salivation occurred in orally-dosed rats. These signs decreased within 5–10 min and test rats assumed a huddled posture.

Beginning 2–3 hr after administration of patulin, some treated rats moved about the cage. At this time, respiratory rates were increased and dyspnoea was observed. A roughened coat, anorexia and arching of the back were prominent signs of toxicity. Within 5 hr after dosing the affected rats either recovered or the signs persisted and progressed to death.

Most of the rats given patulin by the oral and sc routes that died did so within 24 hr of treatment (Table 1). Mortality was delayed in rats given patulin by the ip route; in this group 75% of the deaths occurred 25–72 hr following patulin administration. The single-dose 72-hr $LD_{50}$ values (in mg/kg body

Table 2. *Gross and histopathological alterations in rats given a single dose of patulin (trial I)*

| Route of administration | Patulin dose (mg/kg body weight) | No. of animals with gross alterations* Gastric hyperaemia | Gastric distention | Intestinal hyperaemia | Intestinal distention | No. of animals with microscopic alterations* Gastritis | Enteritis |
|---|---|---|---|---|---|---|---|
| Oral | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 50 | 1 | 5 | 4 | 0 | 7 | 0 |
| | 55 | 1 | 3 | 3 | 1 | 7 | 0 |
| | 60 | 2 | 6 | 4 | 0 | 7 | 0 |
| Subcutaneous | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 8 | 1 | 0 | 1 | 0 | 0 | 0 |
| | 10 | 3 | 0 | 5 | 0 | 0 | 0 |
| | 12 | 2 | 0 | 4 | 0 | 1 | 0 |
| Intraperitoneal | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 9 | 0 | 9 | 5 | 8 | 3 | 0 |
| | 11 | 1 | 5 | 1 | 7 | 4 | 0 |
| | 13 | 2 | 6 | 6 | 4 | 3 | 1 |

*There were ten animals in each group.

weight) and their confidence intervals were as follows: oral, 55·0 (47·0–64·4); sc, 11·0 (9·8–12·3); ip, 10·0 (8·5–11·8).

The principal gross alterations were in the gastrointestinal tract (Table 2). Hyperaemia of the mucosa of the glandular stomach was observed in some rats of all of the patulin-treated groups except one of the groups given patulin ip. In rats that were killed or died immediately before autopsy, distention of the intestinal tract with fluid or gas was present in some rats of all of the groups treated with patulin ip and in one rat in one group treated orally with patulin (Table 2; Fig. 1). Intestinal hyperaemia was observed in some rats of all treated groups.

The principal histopathological alterations were in the stomach. Erosion and ulceration of the mucosa of the glandular portion of the stomach were present in some rats treated by each of the three routes of administration, although they occurred in only one rat in the sc-treated group. These lesions were characterized by submucosal oedema, epithelial cell degeneration and necrosis (Fig. 2). The prevalence of these lesions was not dose related. Peritonitis characterized by fibrin strands and degenerating leucocytes on the intestinal serosa was observed only in ip-dosed rats and was attributed to injection trauma. Other incidental lesions included broncho-pneumonia, bronchiolitis and chronic tracheitis; these alterations were observed in control and treated rats with similar severity and prevalence. Subcutaneous oedema and mild necrosis were present at injection sites in rats administered patulin by the sc route.

Sections of brain, heart, thyroid, thymus, oesophagus, bladder, spleen, testicle, adrenal, pancreas, eye and kidney were histologically unremarkable.

*Trial II*

When multiple doses of patulin were administered the clinical signs, when observed, were similar to those observed in the single-dose study. Some rats died without premonitory signs of toxicity being observed. No differences in clinical signs were found between the groups receiving 50% and those given 75% of the oral $LD_{50}$. Piloerection, dyspnoea, distended abdomen and arching of the back began 12–18 hr after administration. Surviving test rats recovered by day 4 of treatment. An initial loss of body weight was observed in rats given doses of patulin on alternate days at 50 and 75% of the oral $LD_{50}$ (Fig. 3). These groups gained weight by day 4 of patulin administration. Weight gain in all of the patulin-treated groups was at a lower rate than the controls. Mortality was greatest during days 3–7 of the experiment with 56% of deaths occurring during this time (Table 3).

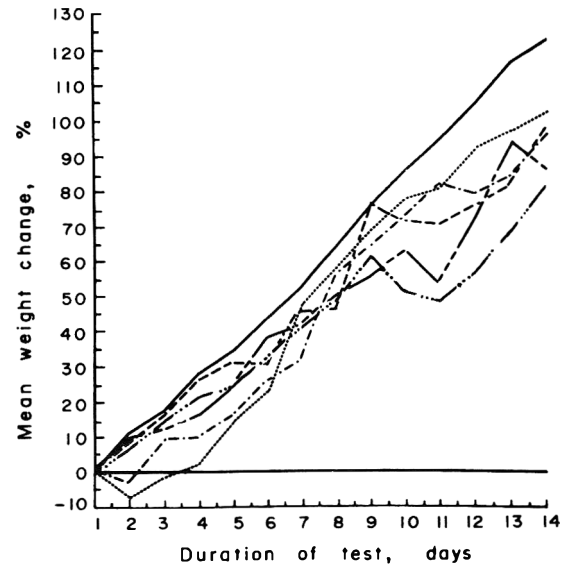


Fig. 3. Mean body weight changes in rats given patulin by gastric intubation daily (D) or on alternate days (AD) at doses of 50 and 75% of the oral $LD_{50}$ (28 and 41 mg/kg body weight, respectively) for 2 wk. The groups were as follows: control (——); 41 mg/kg (D) on days 1–14 (— · · · —); 28 mg/kg (D) on days 1–14 (– – –); 28 mg/kg (D) on days 1–7, 41 mg/kg (D) on days 8–14 (– – ——); 41 mg/kg (AD) on days 1–14 (— · —); 28 mg/kg (AD) on days 1–7, 41 mg/kg (AD) on days 8–14 (· · · · ·).

Gross lesions were principally in the stomach and small intestines. The mucosa of the glandular stomach was reddened and thin-walled due to fluid or gaseous distention. The duodenum and jejunum were reddened and distended by fluid. Histopathological alterations related to compound administration were con-

Table 3. *Mortality, and gross and histopathological alterations in rats given multiple oral doses of patulin over 14 days (trial II)*

| Frequency and size of dose (mg/kg body weight) | | No. of deaths during days* | | | No. of animals with gross alterations* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Wk 1 | Wk 2 | 1–3 | 3–7 | 8–14 | Gastric hyperaemia | Gastric distention | Intestinal hyperaemia | Intestinal distention | No. of animals with gastritis*† |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D28 | D28 | 0 | 5 | 2 | 0 | 7 | 0 | 7 | 9 |
| D41 | D41 | 1 | 5 | 3 | 1 | 7 | 2 | 7 | 7 |
| D28 | D41 | 0 | 5 | 2 | 1 | 5 | 2 | 6 | 9 |
| AD28 | AD28 | 0 | 3 | 4 | 0 | 6 | 0 | 6 | 2 |
| AD41 | AD41 | 1 | 3 | 2 | 0 | 6 | 1 | 6 | 4 |
| AD28 | AD41 | 3 | 3 | 1 | 0 | 6 | 2 | 4 | 3 |

D = Daily AD = On alternate days

*There were ten rats in each group.

†Tissue changes observed by microscopic examination.

Fig. 1. Distention of the stomach and intestines with fluid and gas in a rat given a single oral dose of 55 mg patulin/kg body weight.

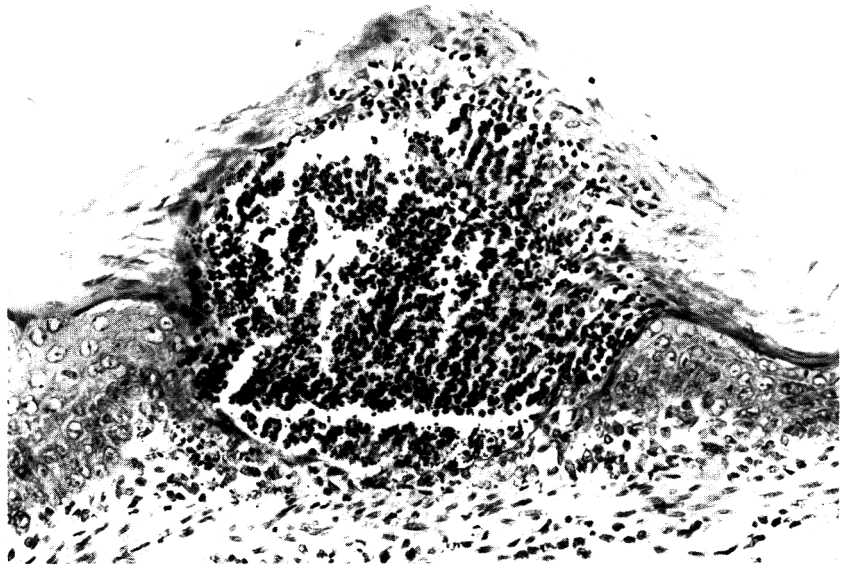

Fig. 2. Focal ulceration and submucosal oedema with squamous cell degeneration, necrosis and focal accumulation of neutrophils in the stomach of a rat given a single ip dose of 11 mg patulin/kg body weight. Haematoxylin and eosin, × 224.

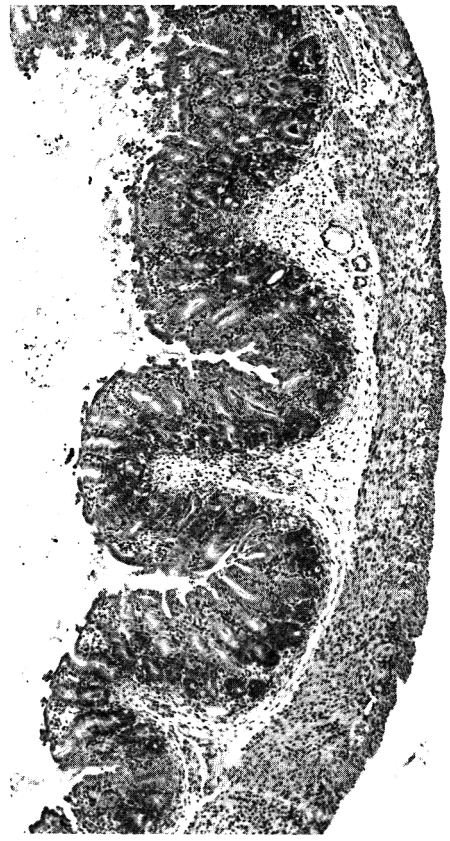

Fig. 4. Erosion, haemorrhages, epithelial cell degeneration and inflammatory cell infiltration of the gastric lamina propria in a rat given multiple oral doses of patulin at 28 mg/kg body weight every day on days 1–7 and 41 mg/kg every day on days 8–14. Haematoxylin and eosin × 35.

Table 4. *Mortality, and gross and histopathological alterations in rats given a single oral dose of patulin and observed for 14 days (trial III)*

| Patulin dose (mg/kg body weight) | No. of deaths during days* | | | No. of animals with gross alterations* | | No. of animals with gastritis*† |
|---|---|---|---|---|---|---|
| | 1–2 | 3–7 | 8–14 | Gastric hyperaemia | Intestinal distention | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 1 | 1 | 0 | 1 | 1 | 1 |
| 41 | 2 | 3 | 0 | 2 | 4 | 0 |

*There were 20 rats in each group.
†Tissue changes observed by microscopic examination.

fined to the stomach. Gastric lesions, found in some rats of all of the treated groups, consisted of erosion and ulceration of the mucosa of the glandular portion of the stomach. These lesions were characterized by epithelial cell degeneration, haemorrhages and neutrophilic and mononuclear cell infiltration of the lamina propria (Fig. 4). The prevalence and severity of the lesions were not dose related, but did occur with greater frequency in animals administered patulin daily.

Incidental histopathological alterations included chronic tracheitis and bronchiolitis. These lesions were observed in both treated and control rats with similar prevalence and severity. Sections of brain, heart, thyroid, thymus, oesophagus, urinary bladder, spleen, testicle, adrenal, pancreas and kidney were histologically unremarkable.

*Trial III*

The clinical signs were similar to those observed in the single- and multiple-dose studies. The time of onset was delayed and the signs were less severe and persisted for a shorter period of time. Roughened coats, arched backs, anorexia, lethargy and dispersion within the cage began 1–2 days after treatment. Clinical signs decreased in severity until day 3 in affected animals or progressed to death. Mortality was dose-related and all deaths occurred during the first 7 days after treatment (Table 4).

Gross lesions included mild hyperaemia and gaseous distention of the stomach and small intestines (Table 4). Histopathological alterations were limited to one animal in the 28-mg/kg group and included focal gastric ulceration with neutrophilic and mononuclear cell infiltration of the lamina propria.

*Trial IV*

Patulin-treated rats showed the same clinical signs as the tested animals in trials I & II. The urine of patulin-treated rats was generally darker than that of control rats. No significant differences between control and treated groups was observed in values of urinary pH, glucose, ketones, bilirubin, urobilinogen and occult blood. Urine taken from patulin-treated rats 24 hr after treatment was occasionally turbid due to the presence of epithelial cells and cellular debris, but generally the urine of both control and treated rats was clear. The urinary volume of the treated group at all time periods was significantly ($P < 0.05$) less than that of the control group (Table 5). Urinary specific gravity in patulin-treated rats was increased at all time periods (Table 5). Urinary protein concentrations (at 3, 12 and 24 hr after treatment were 28, 23 and 24 μg/ml, respectively, in patulin-treated rats and 9, 14 and 8 μg/ml respectively, in controls. However, when total urinary protein was calculated (concentration × volume), no significant differences were evident between the treated and control groups.

Values for blood urea nitrogen, alanine amino transferase and creatinine did not differ significantly between the control and treated groups at the time periods tested. Serum glucose was significantly elevated in patulin-treated rats at 3 and 12 hr after treatment (Table 6). There were fairly wide variations between individual samples taken from control and treated groups at each time interval, but an initial

Table 5. *Volume and specific gravity of urine collected at various times after treatment from control rats and rats given a single oral dose of 41 mg patulin/kg body weight (75% of the oral $LD_{50}$; trial IV)*

| Patulin dose (mg/kg body weight) | Time after treatment (hr) | Specific gravity | Volume (ml) |
|---|---|---|---|
| 0 (control) | 3 | 1·032 ± 0·016 | 2·7 ± 1·9 |
| | 12 | 1·058 ± 0·016 | 4·0 ± 1·2 |
| | 24 | 1·023 ± 0·009 | 14·3 ± 7·1 |
| 41 | 3 | 1·083 ± 0·009 | 0·3 ± 0·48* |
| | 12 | 1·068 ± 0·027 | 1·9 ± 1·6* |
| | 24 | 1·055 ± 0·015 | 6·5 ± 3·3* |

Values are means ± 1 SD for groups of ten rats, and those marked with asterisks differ significantly from the corresponding control value ($P < 0.05$).

Table 6. *Concentrations of glucose and electrolytes in the serum of control rats and rats given a single oral dose of 41 mg patulin/kg body weight (75% of the oral $LD_{50}$) and killed at various times after treatment (trial IV)*

| Patulin dose (mg/kg body weight) | Time after treatment (hr) | Concentration in the serum of | | |
|---|---|---|---|---|
| | | Glucose (mg/ml) | Sodium (mequiv/litre) | Potassium (mequiv/litre) |
| 0 (control) | 0 | 1·04 ± 0·113 | 168 ± 7·2 | 6·5 ± 0·5 |
| | 3 | 1·01 ± 0·19 | 168 ± 21·0 | 6·5 ± 1·1 |
| | 12 | 1·31 ± 0·21 | 164 ± 11·0 | 6·3 ± 0·6 |
| | 24 | 1·09 ± 0·17 | 166 ± 31·0 | 6·1 ± 1·3 |
| 41 | 0 | 1·06 ± 0·142 | 171 ± 17·3 | 6·6 ± 0·9 |
| | 3 | 1·85 ± 0·53* | 151 ± 9·1 | 7·2 ± 1·0 |
| | 12 | 1·71 ± 0·74* | 167 ± 23·0 | 8·2 ± 2·7 |
| | 24 | 1·43 ± 0·39 | 161 ± 17·0 | 6·6 ± 1·4 |

Values are means ± 1 SD for groups of ten rats, and those marked with asterisks differ significantly ($P < 0·05$) from the corresponding control value. There were no significant differences ($P > 0·05$) between the control and treated groups at any time interval in values for blood urea nitrogen, creatinine or chloride concentration, or for alanine–aminotransferase activity.

Table 7. *Plasma-protein concentration and total leucocyte and absolute neutrophil counts for blood taken from control rats and rats given a single oral dose of 41 mg patulin/kg body weight (75% of the oral $LD_{50}$) and killed at various times after treatment (trial IV)*

| Patulin dose (mg/kg body weight) | Time after treatment (hr) | Plasma protein (mg/ml) | Leucocyte count (cells × $10^3$/mm$^3$) | Neutrophil count (cells/mm$^3$)† | |
|---|---|---|---|---|---|
| | | | | Band | Segmented |
| 0 (control) | 0 | 63 ± 2·3 | 7·6 ± 1·23 | 98 ± 101 | 988 ± 246 |
| | 3 | 56 ± 8·9 | 12·0 ± 2·80 | 81 ± 72 | 1156 ± 337 |
| | 12 | 58 ± 3·0 | 13·7 ± 5·00 | 404 ± 574 | 2316 ± 2452 |
| | 24 | 58 ± 3·0 | 14·2 ± 4·90 | 170 ± 119 | 1257 ± 748 |
| 41 | 0 | 66 ± 1·8 | 7·8 ± 1·80 | 142 ± 108 | 1416 ± 1564 |
| | 3 | 49 ± 4·0* | 16·6 ± 5·70* | 240 ± 239 | 6218 ± 3354 |
| | 12 | 43 ± 3·0* | 19·0 ± 7·80* | 289 ± 243 | 7897 ± 2458 |
| | 24 | 44 ± 3·0* | 16·2 ± 4·30 | 200 ± 192 | 3821 ± 2592 |

†Rounded to the nearest whole number.

Values are means ± 1 SD for groups of ten rats and those marked with asterisks differ significantly from the corresponding control values ($P < 0·05$). There were no significant differences ($P < 0·05$) between the control and treated groups at any time interval in values for haematocrit, haemoglobin concentration, total erythrocyte count, and absolute lymphocyte, monocyte and eosinophil counts.

Table 8. *Blood gas values for control rats and rats given a single oral dose of 41 mg patulin/kg body weight (75% of the oral $LD_{50}$) and killed at various times after treatment (trial IV)*

| Patulin dose (mg/kg body weight) | Time after treatment (hr) | pH | $pO_2$ (mm Hg) | $pCO_2$ (mm Hg) | $HCO_3$ (mequiv/litre) | Total $CO_2$ (mmol litre) | Base excess (mequiv/litre) |
|---|---|---|---|---|---|---|---|
| 0 (control) | 0 | 7·25 ± 0·09 | 18 ± 11·7 | 49 ± 4·6 | 20 ± 4·5 | 25 ± 7·9 | −4 ± 1·8 |
| | 3 | 7·25 ± 0·05 | 25 ± 4·1 | 48 ± 3·8 | 21 ± 2·1 | 25 ± 3·0 | −3 ± 4·9 |
| | 12 | 7·27 ± 0·06 | 26 ± 10·1 | 48 ± 4·2 | 21 ± 1·2 | 27 ± 4·2 | −3 ± 1·8 |
| | 24 | 7·28 ± 0·06 | 20 ± 4·4 | 51 ± 3·1 | 22 ± 1·9 | 28 ± 3·6 | −2 ± 2·6 |
| 41 | 0 | 7·21 ± 0·06 | 22 ± 10·4 | 49 ± 8·4 | 22 ± 9·5 | 20 ± 2·7 | −8 ± 3·3 |
| | 3 | 7·43 ± 0·11 | 33 ± 5·0 | 57 ± 17·0* | 39 ± 14·0* | 41 ± 14·0 | +15 ± 14·0 |
| | 12 | 7·31 ± 0·15 | 22 ± 5·0 | 60 ± 18·0* | 29 ± 5·0 | 30 ± 5·0 | +2 ± 7·27 |
| | 24 | 7·40 ± 0·16 | 28 ± 8·0 | 40 ± 10·0 | 24 ± 5·0 | 25 ± 5·0 | −0 ± 8·7 |

Values are means ± 1 SD for groups of ten rats and those marked with asterisks differ significantly from the corresponding control values ($P < 0·05$).

Table 9. *Blood gas values of control rats and rats given a single oral dose of 55 mg patulin/kg body weight (100% of the oral $LD_{50}$) and killed at various times after treatment (trial V)*

| Patulin dose (mg/kg body weight) | Time after treatment (min) | pH | $pO_2$ (mm Hg) | $pCO_2$ (mm Hg) | $HCO_3$ (mequiv/ litre) | Total $CO_2$ (mmol litre) | Base excess (mequiv/ litre |
|---|---|---|---|---|---|---|---|
| 0 (control) | 0 | 7·27 ± 0·04 | 23 ± 9·0 | 47 ± 5·3 | 21 ± 3·8 | 26 ± 5·1 | −3 ± 4·1 |
| | 45 | 7·26 ± 0·06 | 22 ± 7·7 | 47 ± 9·3 | 20 ± 2·6 | 22 ± 2·5 | −6 ± 1·6 |
| | 90 | 7·27 ± 0·08 | 18 ± 8·3 | 42 ± 3·7 | 19 ± 3·7 | 19 ± 2·5 | −9 ± 3·7 |
| | 180 | 7·26 ± 0·06 | 20 ± 7·9 | 42 ± 4·5 | 19 ± 2·8 | 17 ± 3·9 | −9 ± 2·8 |
| | 360 | 7·27 ± 0·06 | 22 ± 6·3 | 42 ± 2·8 | 19 ± 2·7 | 17 ± 2·6 | −8 ± 3·2 |
| 55 | 0 | 7·25 ± 0·05 | 21 ± 9·3 | 46 ± 9·6 | 18 ± 3·5 | 20 ± 3·3 | −8 ± 3·0 |
| | 45 | 7·30 ± 0·06* | 25 ± 11·3 | 50 ± 9·1 | 24 ± 3·6 | 25 ± 3·9 | −4 ± 3·0 |
| | 90 | 7·40 ± 0·08* | 24 ± 6·0 | 52 ± 9·9 | 31 ± 3·8* | 32 ± 4·0 | +5 ± 4·4 |
| | 180 | 7·46 ± 0·07* | 23 ± 6·2 | 57 ± 8·3* | 40 ± 3·8* | 42 ± 6·5 | +12 ± 5·1 |
| | 360 | 7·41 ± 0·06 | 22 ± 3·3 | 61 ± 3·7* | 39 ± 4·4* | 41 ± 4·4 | +9 ± 3·0 |

Values are means ± 1 SD for groups of ten rats and those marked with asterisks differ significantly from the corresponding control values ($P < 0·05$).

decrease in sodium and an increase in potassium were suggested by the values obtained. Serum chloride concentration was not significantly altered.

A significant reduction in plasma protein occurred at 3, 12 and 24 hr after treatment with patulin (Table 7). No differences were observed between treated and control groups for the packed cell volume, haemoglobin and total erythrocyte counts. Total leucocyte counts were significantly elevated in treated rats at 3 and 12 hr after treatment (Table 7). Absolute differential blood counts indicated a neutrophilia in the treated animals at 3, 12 and 24 hours after administration, but there were wide variations between individual samples taken at each time interval. Differences in absolute lymphocyte, monocyte and eosinophil counts were not significant.

The pH values indicated an increase in pH in patulin-treated rats 3 hr after treatment (Table 8), although there was some variation between samples. Bicarbonate ($HCO_3$) values increased significantly in treated animals 3 hr after treatment but were near control values at 12 hr after treatment. Values for $pCO_2$ in patulin-treated animals were increased significantly at 3 and 12 hr and then fell below $pCO_2$ values of control animals at 24 hr after treatment.

*Trial V*

The blood pH values in patulin-treated animals increased significantly with time up to 180 min after treatment but by 360 min the pH value had begun to decrease (Table 9). Blood bicarbonate values in patulin-treated rats were increased significantly by 90 min, peaked at 180 min and remained at peak value until 360 min after treatment (Table 9). Blood $pCO_2$ values were elevated by 45 min after treatment and were significantly elevated after 180 and 360 min (Table 9). Sodium values in patulin-treated rats decreased with time and showed a significant difference from control animals at 360 min after treatment (Table 10). Potassium values tended to increase with time in patulin-treated rats but the values were not statistically different from those of control rats (Table 10).

## DISCUSSION

Patulin was most toxic to the rat when adminis-

Table 10. *Concentrations of electrolytes in serum of control rats and rats given a single oral dose of 55 mg patulin/kg body weight (100% of the oral $LD_{50}$) and killed at various times after treatment (trial V)*

| Patulin dose (mg/kg body weight) | Time after treatment (min) | Concentration (mequiv/litre serum) of | | |
|---|---|---|---|---|
| | | Sodium | Potassium | Chloride |
| 0 (control) | 0 | 179 ± 22·3 | 6·3 ± 1·0 | 101 ± 2·2 |
| | 45 | 178 ± 22·2 | 7·0 ± 1·0 | 106 ± 7·2 |
| | 90 | 173 ± 6·4 | 7·2 ± 0·7 | 110 ± 7·6 |
| | 180 | 174 ± 11·2 | 7·2 ± 0·7 | 110 ± 6·3 |
| | 360 | 172 ± 9·3 | 7·0 ± 0·5 | 102 ± 3·5 |
| 55 | 0 | 175 ± 6·9 | 6·5 ± 0·7 | 108 ± 2·6 |
| | 45 | 165 ± 13·6 | 7·0 ± 1·1 | 102 ± 4·9 |
| | 90 | 160 ± 16·2 | 7·5 ± 1·0 | 98 ± 3·2 |
| | 180 | 156 ± 13·7 | 8·4 ± 1·1 | 96 ± 2·0 |
| | 360 | 148 ± 23·2* | 9·2 ± 1·0 | 96 ± 3·2 |

Values are means ± 1 SD for groups of ten rats and those marked with asterisks differ significantly from the corresponding control value ($P < 0·05$).

tered by the ip route and least toxic by the oral. In these studies, the $LD_{50}$s of patulin by ip and oral routes of administration were greater than those reported by Escoula *et al.* (1977). The $LD_{50}$ for sc administration obtained in this study was lower than that reported by Dailey *et al.* (1977). Such differences may be explained by differences in the strain of rat, the solvent and the source of patulin used or by other variables.

Rats given a single dose of patulin by the oral and sc route died within the 24 hr following administration (Table 1). However, after ip administration deaths were delayed, 13% of deaths occurred 25–48 hr after treatment and 63% occurred 49–72 hr after treatment. Mortality was compound-dependent in groups treated by all routes but was dose-related only in the sc-treated group. Decreased survival time after ip dosing with patulin was observed in mice (Escoula *et al.* 1977; McKinley & Carlton, 1980b) and in hamsters (McKinley & Carlton, 1980a). There is no ready explanation for the observation of delayed mortality in ip-treated rats. In the multiple-dose study (trial II) mortality was greatest 3–7 days after treatment, with 56% of the deaths occurring during this period.

Data from the 14-day multiple-dose study (trial II) indicated no cumulative toxicity. Response to patulin administration was similar at doses of either 50 or 75% of the oral $LD_{50}$ and regardless of the frequency of administration. These results were similar to those reported for the mouse and hamster (McKinley & Carlton, 1980a,b). Freerksen & Bonicke (1951) found no cumulative toxicity in mice after 8 days of patulin treatment, but Broom *et al.* (1944) described cumulative toxicity in mice after 2 wk of patulin treatment. Such differences in opinion as to whether cumulative toxicity occurs after patulin administration remain uninvestigated and unexplained. However, contributory causes could be the source of the patulin used, the rat strain studied and the period of observation.

The initial loss in body weight in two patulin-treated groups (Fig. 3) was attributed to dehydration and was perhaps also caused by reduced feed intake. This early weight loss was followed by recovery of body weight by day 4. The loss in body weight and recovery was similar to that reported in mice (Escoula *et al.* 1977) after a single oral dose of patulin and in mice and hamsters in a similar 14-day study (McKinley & Carlton 1980a,b).

The gross lesions in patulin-treated rats were hyperaemia of the stomach and intestinal tract. This lesion has been reported for the rat (Broom *et al.* 1944; Escoula *et al.* 1977; Katzman *et al.* 1944), mouse (Broom *et al.* 1944; Escoula *et al.* 1977; Katzman *et al.* 1944; McKinley & Carlton, 1980b), hamster (McKinley & Carlton, 1980a) and chicken (Lovett, 1972). Histopathological alterations included gastritis in some rats from all of the groups dosed orally and ip, but in only one rat of the group dosed sc. No dose-effect relationship was evident. Escoula *et al.* (1977) reported ulceration and haemorrhages of the stomach and jejunum of rats and jejunal ulceration in mice treated with patulin. Gastric and intestinal lesions were observed by us in hamsters (McKinley & Carlton, 1980a) and mice (McKinley & Carlton, 1980b). Haemorrhages and necrosis of the lung, liver and kidney and pulmonary oedema described in the mouse (Escoula *et al.* 1977) and the rat (Broom *et al.* 1944; Escoula *et al.* 1977) were not observed by us in the present study. The inflammatory alterations observed in the gastro-intestinal tract may be due to the irritant properties of patulin since we have observed marked irritation in the conjunctival tissues of rabbits following topical application.

When patulin was administered orally to rats at 75% of the oral $LD_{50}$, several clinicopathological values were altered as early as 3 hr after a single dose. Alterations in blood gas measurements in trial IV showed a tendency for the development of a metabolic alkalosis with respiratory compensation. These alterations were confirmed in trial V. Metabolic alkalosis is characterized by an excess of plasma bicarbonate. One mechanism for the development of an elevated plasma bicarbonate is the loss of hydrogen ions through gastric secretions. Normally, gastric hydrogen ions are re-absorbed in the lower intestinal tract. However, if hydrogen ions are not re-absorbed because of vomiting, excessive secretion or intestinal obstruction, a net gain in bicarbonate ions results. The presence of fluid-distended gastro-intestinal tracts suggest that this mechanism could be operative in the patulin-treated rats. Compensatory mechanisms include hypoventilation to conserve $CO_2$ in order to re-establish the bicarbonate–carbonic acid ratio. This compensatory mechanism was evident in trial V where $pCO_2$ values were increased significantly 90 min after a significant elevation of bicarbonate levels.

A marked oliguria was evident throughout the treatment period in patulin-treated rats. Suppression of urine formation was found in rats given patulin sc (Broom *et al.* 1944; Katzman *et al.* 1944). Suppression of urine formation was less severe when patulin was administered by the oral and ip routes (Broom *et al.* 1944) and was found within 4 hr after treatment with patulin (Broom *et al.* 1944). The maintenance of the urine specific gravity above the isosthenuric range indicated that the decreased output was due to decreased perfusion and not to renal disease. Broom *et al.* (1944) reported a decreased $CO_2$ capacity (alkali reserve) of rabbit blood after iv administration of patulin at 5 mg/kg. This is in contrast to the elevated $CO_2$ levels found in our experiments.

Significantly lowered plasma protein levels 12 and 24 hr after patulin treatment indicated that protein as well as hydrogen ions and probably water were lost into the gastro-intestinal tract. The decrease in serum sodium values indicated a loss of sodium in excess of water or isotonic fluid loss and replacement by water intake. Common sites of electrolyte loss are the gastro-intestinal tract and the kidney (Duncan & Prasse, 1977). The gross lesions of fluid distention of the stomach and intestinal tract indicate that this was one possible source of sodium ion loss. A second source was the erythrocytes. In the erythrocytes sodium is normally expelled to the extracellular compartment by an active ATP-dependent pump. After [$^{14}$C]patulin was administered orally to rats, the erythrocyte was found to be the major storage and retention site up to 4 hr after treatment (Dailey *et al.* 1977). Andraud & Andraud (1971) reported that the ATPase of human erythrocytes was inhibited by patulin. The location of patulin in the erythrocytes and its inhibi-

tory action on the sodium pump of the erythrocytic membrane could allow sodium influx into the intracellular fluid of the erythrocyte. However because in the present study the erythrocytic sodium concentration was not determined, this mechanism for serum sodium loss must remain speculative.

Serum potassium tended to increase with time (trial V) but the increases were not significantly different from control values. This tendency for elevation was attributed to interference with the Na–K pump with resultant leakage of potassium ions into the serum. Also, Kahn (1957) reported that patulin inhibited the influx of potassium into incubated cold-stored (potassium-depleted) erythrocytes. Significantly elevated serum glucose levels in patulin-treated rats could not be attributed to stress as a lymphocytopenia in the leucogram did not occur. Broom *et al.* (1944) reported elevated blood glucose in the patulin-treated rabbit but did not determine the cause.

It is speculated that with the elevated serum potassium levels and gastro-intestinal signs, interference with the action of insulin or interference with hormones with secondary effects on insulin such as growth hormone or somatomedin, could have contributed to the elevated serum glucose levels (Duncan & Prasse, 1977; Phillips & Vassilopoulou-Sellin, 1980).

Leucocyte counts were significantly elevated in patulin-treated animals at 3 and 12 hr after treatment and differential blood counts indicated that the elevation was due to neutrophilia. The neutrophilia was attributed to the inflammatory reaction in the gastro-intestinal tract.

The mechanism by which patulin produces its toxic effects (including death) is presently unknown. Patulin reacts with sulphydryl compounds *in vitro* (Atkinson & Stanley, 1943; De Rosnay, Martin-Dupont & Jensen, 1952; Geiger & Conn, 1945) and may react *in vivo* with the sulphydryl groups of critical enzymes (Cavallito & Bailey, 1944; Geiger & Conn, 1945), thus accounting for early acute deaths. The gross and microscopic lesions were possibly related to the antibiotic activity of patulin resulting in a change in the intestinal microflora. Patulin, as an antibiotic, inhibited a wide variety of bacterial organisms (Singh, 1967) but mainly Gram-positive species. The overgrowth of Gram-negative organisms could result in enterotoxaemia and inflammatory changes in the gastro-intestinal tract. These alterations could contribute to and facilitate the metabolic alkalosis by allowing sequestration of hydrogen ions in the gastro-intestinal tract. Antibiotics such as lincomycin hydrochloride and clindomycin caused a marked excessive growth of Gram-negative enteric organisms in hamsters and produced histopathological alterations in the gastro-intestinal tract similar to those produced in our rats (Lusk, Fekety, Silva *et al.* 1978; Small, 1968).

The cause of the metabolic alkalosis is undetermined, but it is possible that subcellular interference with sulphydryl groups in critical enzymes may be a factor by causing increased hydrogen-ion secretion, interfering with hydrogen-ion absorption or disruption of the normal acid-base buffering systems of the body.

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# FAILURE TO PRODUCE HYPERTENSION IN RATS BY CHRONIC EXPOSURE TO CADMIUM

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**Abstract**—Groups of ten male and ten female Sprague–Dawley rats were exposed for 92 and 84 wk, respectively, to increasing concentrations of cadmium (as the chloride) in their drinking-water. The exposure levels of Cd used were 0, 5, 12·6 and 31·5 ppm Cd, and 5 ppm Cd plus certain trace metals. Blood pressure was measured in unanaesthetized animals at regular intervals (46 times in females, 49 times in males) by an indirect method to which the animals were kept conditioned. At the end of the experiments blood pressure was also determined directly under sodium pentobarbital anaesthesia. Although the weight of the kidneys, Cd residues and the severity of histological alterations in the kidneys increased in a dose-related manner, the blood pressure and pulse rate were not significantly affected in any of the experimental groups. There was a dose-dependent decrease in water consumption; all other parameters remained unaffected.

## INTRODUCTION

Cadmium (Cd) is toxic to various organs in experimental animals. Chronic exposure leads to increased renal Cd levels and to diverse histopathological alterations of the kidney (Cooper, 1979; Friberg, Kjellström, Nordberg & Piscator, 1979; Grasso, 1975) and the question has been raised whether this metal might be responsible for hypertension and atherosclerotic heart disease in man. However, although Cd has induced hypertension in experimental animals, observations in man have been inconclusive (Cooper, 1977; Friberg, *et al.* 1979, Gonick, 1978). The work presented here was primarily undertaken to determine the onset of hypertension in rats exposed to increasing Cd concentrations (0–31·5 ppm) in their drinking-water. However, the unexpected results of this study made it necessary to re-evaluate the animal data reported in the literature.

## EXPERIMENTAL

*Animals and feed.* A total of 50 female and 50 male 21-day-old Sprague–Dawley rats (Süddeutsche Versuchstierfarm, Tuttlingen) were randomly allocated to give equal groups and kept under controlled conditions (22–24°C, 45–55% relative humidity, 12 hr light/dark cycles and *c.* 10 room-air changes/hr). They were given feed pellets *ad lib.* According to the manufacturers (Eggersmann, Rinteln) these supplied energy at 12 kJ/g and contained 20% raw protein, 6% raw fat and 4% raw fibre. Their mineral content was as follows: Ca, 1·32%; K, 1·1%; P, 0·92%; Na, 0·39%; Mg, 0·18%; Mn, 80 ppm (min); Zn, 50 ppm (min); Fe, 39 ppm, Cu, 20 ppm (min). The vitamin content per kg of pellets was as follows: vitamin A, 10,000 IU; vitamin D, 1000 IU; vitamin E, 150 mg; vitamin $K_3$, 10 mg; vitamin C, 100 mg; vitamin $B_1$, 20 mg; vitamin $B_2$, 30 mg; vitamin $B_6$, 100 mg, vitamin $B_{12}$, 30 μg, Ca-pantothenate, 40 mg; choline chloride, 1000 mg; folic acid, 5 mg; inositol, 5 mg; biotin. 80 μg. The mean Cd content of the pellets, determined by analysis of seven samples was 0·20 ± 0·10 μg/g dry wt. Drinking-water was given as described below.

*Treatment.* Blood pressure was measured twice in each animal at the age of 32 or 33 and 40 or 41 days (i.e. during wk 1 and 2 of the study) to ensure the elimination of hypertensive animals. None of the rats were found to be hypertensive, and therefore there were ten rats in each group for Cd dosing. All of the rats were given demineralized water *ad lib.* until day 43, when the mean (±SEM) body weights of the males and females were 215 ± 17 and 163 ± 13 g, respectively. Beginning on day 43 Cd (as $CdCl_2$) was added to the drinking-water of three of the groups of each sex at concentrations of 5, 12·6 or 31·5 ppm (concentrations differing by a logarithmic interval of 0·4). Another group of each sex (the controls) were given demineralized water. To the drinking-water of the fifth groups were added 5 ppm Cd as the chloride plus 50 ppm Zn, 10 ppm Mn, 5 ppm Cu and 5 ppm Cr (as the acetates), 1 ppm Co (as the chloride) and 1 ppm Mo (as the molybdate), as recommended by Schroeder & Vinton (1962) and Schroeder (1973).

*Measurements.* Food and water consumption were determined every second or third day and the rats were weighed once a week. Indirect measurements of blood pressure and pulse rate were carried out by fitting a BP-Recorder 8005 (W & W. Basle) to the tail. The measuring system consisted of a Gärtner-type pressure cuff (diameter, 13 mm; length, 28 mm), a piezoelectric crystal as pulse pick-up (diameter, 6·5–8 mm) and a direct recorder with graduated paper (for details see Gerold & Tschirky, 1968). The unanaesthetized rats were immobilized in suitable wire cages covered with aluminium foil to avoid heating up the whole animal. Only the tail was kept outside the cage and was warmed using red light at a distance of 30 cm for about 5 min until pulse-waves were recorded. The pressure in the cuff was increased until

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the pulse waves disappeared and the pressure at which pulse-waves reappeared on reducing the pressure in the cuff was taken as the 'systolic' blood pressure. The results of two measurements performed within 5 min of each other, including the pulse rate counted simultaneously during 6 sec, were averaged. Care was taken to avoid sudden noise, soft music being played from a tape recorder during the whole procedure. The animals were exposed weekly to this procedure throughout the whole observation period and kept conditioned to this except for the compression of the tail. Because ulcers began to develop after 14 wk of observation, the interval between measurements was lengthened to 3 wk without increasing the pressure in the cuff. At the end of the observation period (84 and 92 wk after the start of the study for females and males, respectively) blood pressure was also measured directly in the right carotid artery using a Statham pressure transducer, DC amplifier and a compensograph. Registration was started exactly 20 min after the ip injection of 40 mg pentobarbital/kg body weight.

The rats were killed when moribund and those that survived until the end of the experiment were killed by exsanguination after 60 min of direct measurement of blood pressure, or when they began to wake up from anaesthesia. The Cd concentrations in the tissues were determined according to the methods of Kinrade & Van Loon (1974) and Collet (1975), using atomic absorption spectrophotometry. The detection limit was 0·03 μg Cd/sample and recovery was to 101 ± 2·8% (n = 6). Histological sections (7 μm) were stained by the methods of Goldner (1938).

*Statistical analysis.* Values for body weight, blood pressure and pulse rate were analysed by analysis of variance. Organ-weights related to body weight were transformed to logarithms and subjected to covariance analysis.

## RESULTS

Gross behaviour, body-weight gain and mean food consumption (females, 13–14·5 g/day; males, 19–20 g/day) were not significantly affected in any of the groups. The total Cd-uptake from the food amounted to 1·6–1·7 mg/rat in females and 2·5–2·6 mg/rat in males. A dose-dependent decrease in consumption of drinking-water occurred within the first week of treatment and reached a minimum at wk 40. At the highest Cd concentration mean water consumption, calculated for the whole observation period, was reduced by 40% in females and 37% in males. The vast majority of Cd intake was from drinking-water (Table 1). Some mortality occurred (Table 2), and some tumours (predominantly mammary tumours in females and skin tumours in males) were observed in all groups, but were considered to be unrelated to the treatment. Absolute and relative (fresh) weights of liver, spleen, suprarenals, and ovaries or testes were within the normal ranges; the same was true for heart muscle (Table 2) and aorta, although an increase would have been expected in the presence of hypertension. On the other hand absolute and relative kidney weights showed a tendency to increase (Table 2), the increase being significant ($P < 0·05$) for the right kidney in female rats given 31·5 ppm Cd in their drinking-water, as determined by covariance analysis.

Cd concentrations in the liver and kidneys increased in proportion to the total Cd load (Table 1); there was a highly significant ($P < 0·001$) correlation between Cd uptake and Cd residues in the livers of rats of both sexes (females, $r = 0·94$; males, $r = 0·92$). It should be noted that in the groups treated with 31·5 ppm Cd the total intakes exceeded the acute $LD_{50}$ (54 mg Cd/kg body weight as the chloride, according to Luckey, 1975) by a factor of about 20. Cd residues in liver tissue were higher in female rats

Table 1. *Mean consumption of drinking-water, cadmium intake and cadmium residues in liver and kidney of rats given cadmium in their drinking-water for 82 wk (females) or 90 wk (males)*

| Treatment (ppm Cd in drinking-water) | No. of rats | Water consumption (ml/rat/day) | Total intake *via* drinking-water (mg/rat) | Cd residues (μg/g dry weight) in | |
|---|---|---|---|---|---|
| | | | | Liver | Right kidney |
| | | | **Females** | | |
| 0 | 8 | 37 | 0 | 0·4 ± 0·1 | 1·1 ± 0·3 |
| 5 | 6 | 33 | 98 | 12·1 ± 8·9 | 50·8 ± 26·4 |
| 12·6 | 9 | 29 | 211 | 46·2 ± 11·8 | >80* |
| 31·5 | 8 | 22 | 407 | 136 ± 33 | >100* |
| 5 + minerals† | 9 | 28 | 81 | 12·7 ± 4·1 | 50·9 ± 4·7 |
| | | | **Males** | | |
| 0 | 9 | 44 | 0 | 0·4 ± 0·1 | 1·3 ± 0·4 |
| 5 | 9 | 37 | 120 | 7·3 ± 1·8 | 47·3 ± 16·2 |
| 12·6 | 8 | 37 | 280 | 22·3 ± 9·6 | >60* |
| 31·5 | 8 | 27 | 565 | 67·4 ± 19·8 | >110* |
| 5 + minerals† | 9 | 31 | 100 | 7·3 ± 1·6 | 44·1 ± 11·3 |

*Since concentrations exceeded the linear range of the calibration curve and no more tissue was available, no exact data are given.

†In addition to 5 ppm Cd the rats were given 50 ppm Zn, 10 ppm Mn, 5 ppm Cu and 5 ppm Cr (as the acetates), 1 ppm Co (as the chloride) and 1 ppm Mo (as the molybdate).

Values are means, and means ± 1 SD for Cd residues in liver and kidney for those rats that survived until the end of the experiment.

Table 2. *Mortality and relative organ weights of rats given cadmium in their drinking-water for 82 wk (females) or 90 wk (males)*

| Treatment (ppm Cd in drinking-water) | No. of rats that died* | Relative weights* (% of body weight) of | | |
|---|---|---|---|---|
| | | Heart | Left kidney | Right kidney |
| | | **Females** | | |
| 0 | 2 | 0·25 | 0·26 | 0·26 |
| 5 | 4 | 0·26 | 0·26 | 0·26 |
| 12·6 | 1 | 0·24 | 0·28 | 0·28 |
| 31·5 | 2 | 0·25 | 0·29 | 0·30 |
| 5 + minerals† | 1 | 0·25 | 0·26 | 0·27 |
| | | **Males** | | |
| 0 | 1 | 0·25 | 0·28 | 0·27 |
| 5 | 1 | 0·24 | 0·29 | 0·29 |
| 12·6 | 2 | 0·26 | 0·33 | 0·33 |
| 31·5 | 2 | 0·25 | 0·32 | 0·32 |
| 5 + minerals† | 1 | 0·25 | 0·29 | 0·28 |

*There were ten rats in each group. The relative organ weights are means for the survivors of each group.

†In addition to 5 ppm Cd the rats were given 50 ppm Zn, 10 ppm Mn, 5 ppm Cu and 5 ppm Cr (as the acetates), 1 ppm Co (as the chloride) and 1 ppm Mo (as the molybdate).

than in males at all Cd dose levels (Table 1), a phenomenon previously described by Stonard & Webb (1976). No convincing explanation can be given for this observation. There was no such difference between the sexes in Cd accumulation in the kidney (Table 1).

No histological alterations indicative of chronic hypertension were observed in cardiac or aortic tissues. In addition to age-dependent changes, which were somewhat more pronounced in male rats, the renal tissues of treated rats showed cloudy swelling of the epithelium of the proximal convoluted tubules with vacuolar degeneration and thickening of the walls of the interlobular arteries. The severity of these changes was greatest in the groups given 12·6 and 31·5 ppm Cd.

Blood pressure, measured indirectly during the first 14 wk of treatment, is shown in Fig. 1. Regardless of sex and treatment an increase of about 10 mmHg in females and 20 mmHg in males was observed in all groups. This increase parallels the increase in body weight during that period. There were significant correlations between body weight and blood pressure (values transformed into logarithms: $r = 0{\cdot}42$–$0{\cdot}57$ and $0{\cdot}57$–$0{\cdot}71$ for females and males, respectively). Increasing blood-pressure values have also been described during childhood by Maclain (1976). These data are unlikely to be artefacts produced for example by the increasing thickness of the tissues compressed by the cuff. Blood-pressure values remained fairly constant in both females and males after wk 24. Although mean blood-pressure values were slightly increased by 5–10 mmHg throughout the study and on three occasions by 15 mmHg in females exposed to the two highest Cd concentrations (once in the 12·6-ppm group and twice in the 31·5-ppm group), no significant differences between the groups were demonstrable by analysis of variance at any time interval. The same was true for the pulse rate which remained constant in all groups throughout the whole observation period.

At the end of the experiment (84 wk for females and 92 wk for males) arterial blood pressure was measured directly in a carotid artery at different times following pentobarbital anaesthesia. The results are summarized in Table 3. There were no significant differences between the groups at any time, even when taking

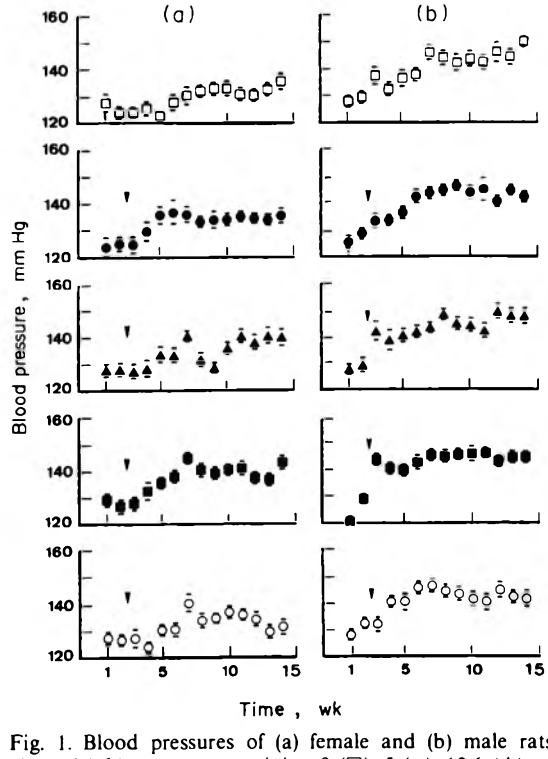


Fig. 1. Blood pressures of (a) female and (b) male rats given drinking-water containing 0 (□), 5 (●), 12·6 (▲) or 31·5 (■) ppm Cd or 5 ppm Cd plus certain minerals (○). For details see experimental section. Cd dosing was begun at wk 3, as indicated by arrows; rats were given demineralized water during wk 1 and 2. Values are means and range bars indicate the SEM.

Table 3. *Blood pressures determined directly in the carotid artery of rats at intervals after anaesthesia with 40 mg pentobarbital/kg body weight*

| Treatment (ppm Cd in drinking-water) | Blood pressure (mmHg) determined at an interval after anaesthesia of | | | |
|---|---|---|---|---|
| | 20 min | 30 min | 45 min | 60 min |
| | **Females** | | | |
| 0 | 121 ± 10 (6) | 131 ± 6 (6) | 114 ± 24 (6) | 102 ± 31 (5) |
| 5 | 106 ± 21 (5) | 128 ± 30 (5) | 134 ± 30 (5) | 145 ± 21 (5) |
| 12·6 | 99 ± 26 (8) | 109 ± 22 (9) | 103 ± 22 (9) | 113 ± 28 (9) |
| 31·5 | 111 ± 23 (6) | 128 ± 14 (6) | 113 ± 26 (6) | 120 ± 30 (6) |
| 5 + minerals* | 107 ± 15 (7) | 118 ± 11 (9) | 103 ± 26 (8) | 113 ± 22 (8) |
| | **Males** | | | |
| 0 | 134 ± 15 (8) | 137 ± 13 (8) | 139 ± 15 (8) | 114 ± 19 (8) |
| 5 | 119 ± 25 (8) | 125 ± 26 (8) | 133 ± 16 (8) | 138 ± 16 (7) |
| 12·6 | 114 ± 31 (7) | 125 ± 32 (7) | 128 ± 35 (7) | 141 ± 27 (7) |
| 31·5 | 133 ± 16 (7) | 139 ± 17 (7) | 141 ± 21 (7) | 141 ± 17 (6) |
| 5 + minerals* | 123 ± 24 (9) | 117 ± 29 (9) | 126 ± 39 (9) | 135 ± 20 (8) |

*In addition to 5 ppm Cd the rats were given 50 ppm Zn, 10 ppm Mn, 5 ppm Cu and 5 ppm Cr (as the acetates), 1 ppm Co (as the chloride) and 1 ppm Mo (as the molybdate).

Values are means ± 1 SD for the number of rats indicated in brackets.

into account the area under the pressure curves in the interval from 20 to 60 min. It should be noted, however, that the blood pressure tended to increase in most groups after 40 min, especially in males which woke up at that time. This tendency was less noticeable in females which slept much longer (> 150 min) and displayed sudden falls of blood pressure of up to 40 mmHg at irregular times, these falls lasting for 10–15 min.

## DISCUSSION

On reviewing the relevant literature on the interactions of orally administered Cd and blood pressure in rats we found 11 original articles reporting the development of hypertension when Cd (as the chloride or acetate) was added to drinking-water. Schroeder and his co-workers (Schroeder, 1964; Schroeder & Buckman, 1967; Schroeder, Nason & Balassa, 1967; Schroeder, Nason & Mitchener, 1968; Schroeder & Vinton, 1962) used concentrations of 0–5 ppm Cd together with certain minerals. Therefore in the present studies in addition to 5 ppm Cd we gave one group of female and one of male rats these trace elements together with 5 ppm Cr as recommended by Schroeder (1973). Similar experimental conditions were chosen by Perry's group (Perry & Erlanger, 1974 & 1978; Perry, Erlanger & Perry, 1976 & 1977a,b) except that in the first two experiments Cd concentrations ranged from 0 to 50 ppm. In most of these studies female Long–Evans rats were used because according to Schroeder & Vinton (1962 & 1967) and Schroeder (1964) these animals are more sensitive to developing Cd-induced hypertension than are males. Except in the first study by Schroeder's group (Schroeder & Vinton, 1962) in which blood-pressure measurements were made every 3 wk, intervals of 3 months or longer were used both by Schroeder *et al.* and Perry *et al.* Therefore in their experiments the animals were not conditioned to the procedure and had to be anaesthetized, usually with sodium pentobarbital given ip when blood pressure was measured indirectly. Schroeder (1962) used a dose of 45 mg/kg, but in later papers the dose is not stated. Perry *et al.* (1974, 1976, 1977*a* & 1978) used 0·35 mg/kg and in another experiment (Perry *et al.* 1977b) 25 mg sodium pentothal/kg. The animals had to be warmed up in warming boxes; however the exact time after starting anaesthesia at which the tests were performed was not stated. In Schroeder's studies hypertension reached values up to + 122 mmHg, control values being sometimes surprisingly low at 84 ± 5·7 mmHg (Schroeder & Buckman, 1967). Perry *et al.* (1974, 1976, 1977a,b & 1978) reported that the degree of hypertension was less pronounced (differences of + 10 to + 33 mmHg). The statistical analysis of the results of both groups of workers was done by Student's *t* test or was not described in detail.

Revis (1978), using male Sprague–Dawley rats, also reported increased blood pressure 60 days after exposure to 3 ppm Cd in the drinking-water. Blood pressure was measured directly in a femoral artery, again under pentobarbital anaesthesia (40 mg/kg).

In eight other studies carried out on male or female rats of various strains (Long–Evans, OSU, Wistar, Sprague–Dawley) no Cd-induced hypertension had been observed. It must be admitted that in six of these studies the observation periods were only 28 wk or less (Frickenhaus, Lippal, Gordon & Einbrodt, 1976; Kotsonis & Klaassen, 1977 & 1978; Loeser & Lorke, 1977; Ohanian, Iwai, Leitl & Tuthill, 1978; Schroeder, Whanger & Weswig, 1973). Doyle, Bernhoft & Sandstead (1975) used pentobarbital anaesthesia in a 320-day experiment, but standardized the interval after induction of anaesthesia for making their measurements. Kotsonis & Klaassen (1978) used urethane for anaesthesia, which in our experience affects blood pressure only minimally, and Ohanian *et al.* (1978) used ether. The results of the present study agree best with those of Eakin, Schroeder, Whanger & Weswig (1980) who exposed OSU rats to Cd concentrations of 0, 10 and 20 ppm for 20 months. They

measured blood pressure at intervals of 4 wk for the first 24 wk and then at 8-wk intervals, using pentobarbital anaesthesia (100 mg/kg) only once at 20 or 40 wk, and detected no signs of hypertension.

Consideration of all of the animal data reported justifies the conclusion that prolonged Cd intake does not produce any marked hypertension in rats. Minor increases in blood pressure of up to 15 mmHg may occur; such increases were observed occasionally in female rats in the present study but no gross or microscopically detectable changes typical of hypertension were observed in any of the organs examined. If optimal experimental conditions are chosen (i.e. keeping the animals conditioned to the test procedure, avoiding pentobarbital anaesthesia, minimizing the stress of warming-up, giving sufficient Cd to show dose-dependent alterations without inducing signs of acute toxicity) and if appropriate statistical methods are applied, it must remain doubtful whether the observed changes in blood pressure ever become significant in the absence of additional factors such as for example an increased supply of NaCl or the use of hypertension-sensitive rats (Ohanian *et al.* 1978).

Nevertheless the fact that Cd accumulates in the liver and more particularly in the kidneys, in which morphological lesions are induced, calls for close attention in the future. Further work is needed to establish the exact concentrations at which these changes are induced and to study their reversibility.

We have carried out additional experiments that have shown that during 16 wk the consumption of drinking-water was not affected by 50 ppm Cd added to the diet instead of to the drinking-water, and so the observed reduced water consumption may not be due to central nervous reactions and may be of minor toxicological importance.

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# HEPATIC MICROSOMAL ENZYME INDUCTION BY AROCLORS 1248 AND 1254 IN CYNOMOLGUS MONKEYS

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**Abstract**—Aroclors 1248 (2 mg/kg body weight) and 1254 (5 mg/kg body weight) produced a 3-methylcholanthrene type of mixed-function oxidase induction, in the livers of adult female cynomolgus monkeys after 69–122 days of dosing, when the animals were moribund. Both Aroclor 1248 and 1254 produced a mixed pattern of induction in mouse liver 66 hr after ip treatment with 1000 mg/kg body weight including changes characteristic of 3-methylcholanthrene-type induction. However the same dose of Aroclor 1016 did not produce 3-methylcholanthrene-type enzyme induction in mice. The results in adult monkeys are consistent with the hypothesis that PCB isomers capable of inducing aryl hydrocarbon hydroxylase and causing a 'blue' shift in the cytochrome peak, are associated with increased toxicity. However the lack of response with Aroclor 1016 in mice suggests that the reported toxicity of this aroclor to infant monkeys may not result from isomers producing a 3-methylcholanthrene-like effect.

## INTRODUCTION

Numerous studies have shown that the PCB compounds can elicit a variety of toxic effects in several animal species. Recent reports have revealed an apparent sensitivity of non-human primates to PCB intoxication (Allen, Barsotti, Lambrecht & Van Miller, 1979; Altman, New, McConnell & Ferrell, 1979; Barsotti, Marlar & Allen, 1976).

One effect, common to most species tested, is the PCB-mediated increase in hepatic microsomal MFO activity. It is known that individual isomeric components (congeners) of a commercial PCB mixture are able to produce either a cytochrome *P*-450 ('phenobarbital-like') effect or a cytochrome *P*-448 ('3MC-like') effect, or both effects simultaneously, as judged by increases in the activity of enzymes acting on selected substrates, and changes in the peak location of the carbon-monoxide-bound cytochrome (Goldstein, Hickman, Bergman *et al.* 1977; Parkinson, Cockerline & Safe, 1980; Yoshihara, Kawano & Yoshimura, 1979).

Increased enzyme activity *per se* might provoke deleterious effects through modulation of endogenous hormone levels, or *via* enhanced net conversion of certain PCB congeners to a chemically reactive species capable of eliciting a tissue-damaging effect. Moreover, there appears to be a correlation between observed toxicity and the ability of various chemicals to elicit a 3MC-type response in MFO enzyme activity.

Nebert (1978a), and Poland & Glover (1980), have suggested that the expression of toxicity may be part of the pleiotropic response that occurs when certain PCB isomers initiate the sequence of steps that ultimately results in increased synthesis of specific cytochrome components and an increase in aryl hydrocarbon hydroxylase (AHH) activity.

With this in mind, the MFO activity was determined in liver microsomal preparations obtained from cynomolgus monkeys that had received the PCB mixtures Aroclor 1248 and Aroclor 1254.

## EXPERIMENTAL

Adult female cynomolgus monkeys (*Macaca fascicularis*) were given Aroclor 1254 (5 mg/kg; lot no. 634, Monsanto, St. Louis, MO) or Aroclor 1248 (2 mg/kg; from J. R. Allen, University of Wisconsin, WI, USA). The PCBs were dissolved in corn oil and an aliquot of this solution was emulsified in approximately 45 ml of 0·25% gelatin in apple juice. There were two monkeys on each aroclor and two controls, each animal receiving the appropriate apple juice–PCB mixture (or vehicle control) three times a week (equivalent to 2 or 5 mg/kg/day). Dosing was continued until the animals became moribund (69–122 days) whereupon they were autopsied. Liver samples were obtained and microsomal fractions were prepared immediately (Iverson, 1976).

Male mice (HPB black, C57-derived) were dosed ip with 1000 mg Aroclor 1248, 1254 or 1016/kg in corn oil, and killed 66 hr after treatment. Washed microsomes were prepared as above. There were three animals in each treatment group.

Enzyme activities were determined as follows: aminopyrine *N*-demethylase by the method of Cochin & Axelrod (1959); acetanilide hydroxylase according to Krisch & Staudinger (1961); benzo[*a*]pyrene hydroxylase (aryl hydrocarbon hydroxylase, AHH) by the method of Nebert (1978b). The 3-hydroxybenzo-[*a*]pyrene standard was the generous gift of the NCI Carcinogenesis Research Program. Ethoxyresorufin was synthesized and determined by the method of

*Abbreviations*: PCB = Polychlorinated biphenyl; MFO = Mixed-function oxidase; 3MC = 3-Methylcholanthrene; AHH = Aryl hydrocarbon hydroxylase.

Table 1. *Effect of Aroclors 1248 and 1254 on the liver microsomal enzyme activity of cynomolgus monkeys**

| Treatment | | Aminopyrine *N*-demethylase (nmol/min/ mg Pr)* | Acetanilide hydroxylase (nmol/min/ mg Pr)* | Aryl hydrocarbon hydroxylase (nmol/min/ mg Pr)* | Ethoxycoumarin deethylase (nmol/10 min/ mg Pr)* | Ethoxyresorufin deethylase (nmol/10 min/ mg Pr)* | *P*-450 level (nmol/mg Pr)* | *P*-450 peak (nm) |
|---|---|---|---|---|---|---|---|---|
| Control | | 3·35 | 3·74 | 0·36 | 7·85 | 0·83 | 1·061 | 450·6 |
| | | 3·70 | 3·61 | 0·34 | 5·56 | 0·67 | 1·232 | 450·4 |
| | Mean... | 3·53 | 3·68 | 0·35 | 6·71 | 0·75 | 1·147 | 450·5 |
| Aroclor 1248 | | 5·48 | 6·02 | 0·72 | 13·60 | 5·41 | 2·239 | 449·0 |
| | | 5·47 | 5·41 | 0·96 | 13·60 | 4·00 | 2·354 | 449·2 |
| | Mean... | 5·48 | 5·72 | 0·84 | 14·60 | 4·71 | 2·297 | 449·1 |
| | % of control... | 155 | 155 | 240 | 218 | 630 | 200 | |
| Aroclor 1254 | | 3·07 | 5·24 | 0·72 | 12·59 | 4·02 | 1·531 | 449·4 |
| | | 3·40 | 6·92 | 0·46 | 11·40 | 5·10 | 2·558 | 449·3 |
| | Mean... | 3·23 | 6·08 | 0·59 | 12·20 | 4·56 | 2·045 | 449·4 |
| | % of control... | 92 | 165 | 185 | 182 | 610 | 182 | |

Pr = Protein

*All enzyme values are reported as nmol product formed/min or 10 min/mg protein. Cytochrome *P*-450 levels are reported as nmol cytochrome/mg protein.

Table 2. *Effect of Aroclors 1016, 1248 and 1254 on the liver microsomal enzyme activity of HPB black mice**

| | | Aminopyrine *N*-demethylase (nmol/min/ mg Pr)* | Acetanilide hydroxylase (nmol/min/ mg Pr)* | Aryl hydrocarbon hydroxylase (nmol/min/ mg Pr)* | Ethoxycoumarin deethylase (nmol/10 min/ mg Pr)* | Ethoxyresorufin deethylase (nmol/10 min/ mg Pr)* | *P*-450 level (nmol/mg Pr) | *P*-450 peak (nm) |
|---|---|---|---|---|---|---|---|---|
| Control | | 5·29 ± 0·61 | 4·79 ± 0·57 | 0·85 ± 0·07 | 2·11 ± 0·27 | 0·61 ± 0·16 | 0·539 ± 0·025 | 450·1 ± 0·1 |
| Aroclor 1248 | | 14·84 ± 1·63 | 28·62 ± 2·17 | 3·73 ± 2·17 | 43·13 ± 11·98 | 24·17 ± 1·23 | 1·975 ± 0·162 | 449·2 ± 0·1 |
| | % of control... | 281 | 597 | 439 | 2044 | 3898 | 366 | |
| Aroclor 1254 | | 16·21 ± 1·04 | 40·88 ± 1·48 | 4·04 ± 0·39 | 68·23 ± 3·13 | 29·13 ± 2·72 | 2·438 ± 0·047 | 449·0 ± 0·1 |
| | % of control... | 306 | 823 | 475 | 3234 | 4698 | 452 | |
| Aroclor 1016 | | 10·22 ± 0·98 | 12·25 ± 0·85 | 0·99 ± 0·11 | 14·71 ± 3·79 | 1·62 ± 0·09 | 1·066 ± 0·054 | 450·2 ± 0·1 |
| | % of control... | 193 | 256 | 116 | 697 | 261 | 198 | |

Pr = Protein

*All enzyme values are reported as nmol product formed/min or 10 min/mg protein. Cytochrome *P*-450 levels are reported as nmol cytochrome/mg protein. Values are means ± SEM for groups of three animals.

Burke & Mayer (1974). Ethoxycoumarin was synthesized and determined by the method of Ullrich & Weber (1972). Cytochrome *P*-450 was determined by the method of Omura & Sato (1964) using an Aminco DW-2a spectrophotometer. Protein was assayed by the biuret method of Gornall, Bardawill & David (1949).

Microsomal incubations were conducted in a 37°C shaking bath using scintillation vials containing microsomes (1 mg/ml), tris buffer, pH 7·4 (50 mM), glucose-6-phosphate (3 mM), NADP (0·6 mM), glucose-6-phosphate dehydrogenase (2 units), $MgCl_2$ (5 mM) and substrate. With ethoxycoumarin and ethoxyresorufin, NADPH was substituted for the NADPH generating system. NADPH and components of the generating system were obtained from Sigma Chemical Co., St Louis, MO.

## RESULTS AND DISCUSSION

After 69–122 days of dosing, the monkeys in both treatment groups exhibited, to varying degrees, laboured breathing, ataxia and tremors, and were considered moribund. A complete description of the pathological findings will be published elsewhere. Table 1 lists the results obtained with the monkeys. There were two animals in each group and the percentage change in the average value of the activities compared with that of the controls is given in the table. With Aroclor 1248 all the measured activities were increased when calculated on a protein basis. Since the level of cytochrome *P*-450 was also increased, calculation of activity on a *P*-450 basis would have shown increases in the activities of AHH, ethoxycoumarin deethylase and ethoxyresorufin deethylase only. On a protein basis with Aroclor 1254, all of the activities with the exception of that of *N*-demethylase were increased. Calculated on a *P*-450 basis only the ethoxyresorufin deethylase activity is increased. The increases in these parameters indicate that the enzyme systems remained viable despite the moribund state of the treated monkeys.

Few studies have been reported using monkey liver to determine changes in AHH activity. Thorgeirsson, Sakai & Adamson (1978) showed that a single 80 mg/kg dose of 3-MC administered to rhesus monkeys produced a typical increase in AHH activity and a 'blue' shift in the carbon-monoxide-bound cytochrome peak. Control values for AHH activity have been determined in cynomolgus monkeys by Chasseaud, Down, Grover *et al.* (1980) and these values are almost identical to those found in the present study. The six-fold increase in ethoxyresorufin deethylase activity and the minimum 1·1 nm 'blue' shift in *P*-450 peak location, suggest that the aroclors do produce a 3 MC-like pattern of enzyme induction in cynomolgus monkeys. These criteria have also been used for the detection of the induction of different types of cytochrome *P*-450 in other animal species (Kohli, Philpot, Albro & McKinney, 1980).

Table 2 shows the effects of Aroclors 1248, 1254 and 1016 on mouse liver MFOs. With the greatly increased dose level, it is clear that both the 1248 and 1254 mixtures contain PCB congeners that elicit pronounced effects on ethoxyresorufin deethylase, AHH, and produce a 'blue' shift in the location of the *P*-450 peak, results similar to those found in the monkeys. The increase in aminopyrine *N*-demethylase activity reveals that 'phenobarbital-like' effects are also produced by these Aroclors. In contrast to these data, the Aroclor 1016 results reveal only a minimal change in the parameters characteristic of '3MC-like' effects; this is consistent with previous results (Alvares, Fischbein, Anderson & Kappas, 1977; Iverson, Villeneuve, Grant & Hatina, 1975) indicating that the PCB components responsible for the '3MC-like' effects are absent or in an extremely low concentration in this Aroclor. Recent analytical work (Albro & Parker, 1979) has shown that there is an absence of specific congeners in 1016 and dibenzofuran contaminants were not detected (detection limit 5 ppb).

The results suggest that Aroclors 1248 and 1254 elicit an MFO response in cynomolgus monkeys similar to that observed in mice. Since the monkeys become moribund after 69–122 days at 2 mg Aroclor 1248 and 5 g Arocolor 1254/kg, it would appear that they are more sensitive than the rat which can tolerate either of these Aroclors at 100 ppm in the diet ($\simeq$8 mg/kg) for 1 year (Allen, Carstens & Abrahamson, 1976). Both species can also exhibit similar MFO changes and it would appear that if toxicity is to be closely related to MFO response there must be other factors operating as well. Perhaps there is a species difference in the structure of the receptor that binds with the inducers prior to initiating the induction of the enzymes.

Recent work by McNulty, Becker & Cory (1980), showed that, at equivalent dose levels, the AHH inducer 3,3′, 4,4′-tetrachlorobiphenyl rapidly produces typical toxic symptoms in the rhesus monkey while the 2,2′, 5,5′-isomer which does not induce AHH does not produce toxic symptoms within the same time period. These data as well as the results of the present study, and prior work by Barsotti *et al.* (1976), are at least consistent with the hypothesis that PCB isomers capable of producing a 'methylcholanthrene-like' effect are more toxic.

Aroclors 1248 and 1254 are known to contain isomers capable of AHH induction. Since 1248 at 2 mg/kg appears to be as effective at induction as 1254 at 5 mg/kg, it seems likely that the former PCB is more toxic. However, a direct comparison of potency is not possible without additional dose-response data since it is possible that the effects of Aroclor 1254 peak at 2 mg/kg.

While a close, and growing, correlation between enzyme induction by selected PCB isomers and toxicity is apparent in several species, this may not be the case for PCB effects observed with infant monkeys allowed to nurse from mothers who were fed diets containing PCBs during pregnancy. A preliminary report (Barsotti & Allen, 1980) has indicated that nursing infants exhibit the same signs of toxicity (low birth weight, hyperpigmentation) when the mothers had been given Aroclor 1248 or Aroclor 1016 at the same levels. As indicated earlier, there is analytical and toxicological evidence that Aroclor 1016 contains neither sufficient dibenzofuran contaminants nor sufficient PCB isomers to elicit the 'methylcholanthrene-like' response that is observed with Aroclor 1248 (or 1254). Although this does not refute the proposed correlation of certain toxic manifestations with enzyme

response in adult animals, it does suggest that the signs of toxicity in infants may not necessarily be correlated with the content of PCB isomers eliciting an increase in AHH activity.

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# SHORT PAPERS

# ACUTE ORAL TOXICITY OF INORGANIC COBALT COMPOUNDS IN RATS

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**Abstract**—Eight cobalt compounds were administered to rats by gastric intubation, and the following $LD_{50}$ values (in mg anhydrous compound/kg body weight) were determined: cobalt(II) fluoride 150, cobalt(II) oxide 202, cobalt(II) phosphate 387, cobalt(II) bromide 406, cobalt(II) chloride 418, cobalt(II) sulphate 424, cobalt(II) nitrate 434 and cobalt(II) acetate 503. After administration of the cobalt compounds, body temperatures decreased by 2·5–7·5°C. The liver, heart and kidneys of rats given cobalt(II) fluoride or oxide were examined microscopically. Hyperaemia, haemorrhage and cytoplasmic changes were noted, while the kidney glomeruli were very rich in cells and basal membranes were thickened. Cells of the proximal tubules were swollen and showed vacuolization and degeneration. In the hearts of some rats proliferative and oedematous interstitial tissue and swollen muscle fibres were observed, and focal degeneration, vacuolization and necrosis associated with disappearance of the cross striations were also noted.

## Introduction

Although inorganic cobalt compounds are widely used industrially, for example as pigments for ceramics, in fast-drying paints and varnishes, in printing-inks, in storage-battery electrodes and as oxidation catalysts in drying oils, there is little information on their acute toxicity. The oral $LD_{50}$ value of cobalt(II) oxide in rats has been reported as 1700 mg/kg body weight (Smyth, Carpenter, Weil *et al.* 1969) and that of cobalt(II) chloride as 180 mg/kg (Christensen & Luginbyh, 1974). Since these values show an unexpected divergence, the acute toxicity of eight cobalt compounds, including the cobalt(II) oxide and chloride, was investigated in the study reported here.

## Experimental

*Test compounds.* Cobalt(II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$), cobalt(II) sulphate heptahydrate ($CoSO_4.7H_2O$), cobalt(II) acetate tetrahydrate ($Co(CH_3COO)_2.4H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$) and cobalt(II) fluoride ($CoF_2$) were obtained from E. Merck AG, Darmstadt, FRG, cobalt(II) bromide ($CoBr_2$) and cobalt(II) oxide (CoO) from Alfa Divn, Ventron Corp., Danvers, MA, USA, and cobalt(II) phosphate octahydrate ($Co_3(PO_4)_2.8H_2O$) from ICN Pharmaceuticals, New York, USA. Apart from cobalt(II) bromide and oxide, which had a purity of 95%, these cobalt compounds were 99% pure.

*Animals and diet.* Wistar rats (R.v:TOX[M]) obtained from the colony of the Animal Breeding Unit, National Institute of Public Health, Bilthoven, and weighing approximately 100 g, were caged singly or in pairs of the same sex and dose group, in wire cages at a room temperature of 22–25°C and relative humidity of 35–55%. The animals were provided with a semi-purified diet, Muracon-SSP Tox (Trouw Ltd, Putten) and drinking-water *ad lib.*

*Experimental design.* Each compound was tested in five groups of five male and five female rats, given the doses reported in Table 1. The compounds were administered by gastric intubation in a single dose dissolved in distilled water or suspended by means of an Ultra-Turrax (Janke u. Kunkel GmbH, Staufen, FRG) in a 1% carboxymethylcellulose solution. Signs of reaction and deaths were recorded for 10 days, and the rectal temperature was measured in all surviving rats 1·5, 24 and 48 hr after administration of the cobalt compound. In the groups dosed with cobalt(II) fluoride or oxide, microscopic examination of the heart, liver and kidneys was carried out on all rats surviving for 48 hr or more after treatment (i.e. on those dying between day 2 and day 10 or killed at the end of the 10-day observation period).

*Statistics.* The oral $LD_{50}$ values, for male and female rats combined, were calculated according to the method of 'maximum likelihood' of Finney (1971).

## Results

### *Clinical observations*

The highest dose levels of all the cobalt compounds caused sedation and diarrhoea. Rats given cobalt(II) sulphate or chloride also showed tremors and convulsions prior to death. Respiratory disturbances were apparent in rats given the acetate. Animals in the cobalt(II) fluoride group did not show any of these effects. In all animals given cobalt compounds, a de-

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Table 1. *Acute oral toxicity of cobalt compounds in Wistar rats*

| Test compound | Molecular weight | Dose administered* (mg/kg body weight) 1 | 2 | 3 | 4 | 5 | $LD_{50}$ values (mg/kg body weight) Of cobalt compound tested† | Calculated for Anhydrous compound | Cobalt(II) ion |
|---|---|---|---|---|---|---|---|---|---|
| $CoF_2$‡ | 96·9 | 70 | 112 | 180 | 290 | 460 | 150 (115–195) | 150 | 91 |
| CoO‡ | 75·0 | 200 | 300 | 450 | 675 | 1010 | 202 (136–300) | 202 | 159 |
| $Co_3(PO_4)_2.8H_2O$‡ | 511·0 | 300 | 450 | 675 | 1000 | 1500 | 539 (425–683) | 387 | 187 |
| $CoBr_2$§ | 218·7 | 200 | 300 | 450 | 675 | 1010 | 406 (330–500) | 406 | 109 |
| $CoCl_2.6H_2O$§ | 238·0 | 500 | 600 | 720 | 864 | 1137 | 766 (677–867) | 418 | 190 |
| $CoSO_4.7H_2O$§ | 281·0 | 450 | 675 | 1000 | 1500 | 2250 | 768 (594–995) | 424 | 161 |
| $Co(NO_3)_2.6H_2O$§ | 291·1 | 450 | 675 | 1000 | 1500 | 2250 | 691 (526–907) | 434 | 140 |
| $Co(CH_3CO_2)_2.4H_2O$§ | 249·1 | 250 | 375 | 560 | 840 | 1260 | 708 (569–880) | 503 | 168 |

*Each dose level was administered by gavage to a group of five males and five females.
†With 95% confidence interval in brackets.
‡Vehicle: 1% carboxymethylcellulose.
§Vehicle: distilled water.

crease in body temperature was recorded, varying from 2·5–7·5°C. These temperature reductions were time- and dose-related, as demonstrated in Fig. 1 for cobalt(II) fluoride and oxide.

*$LD_{50}$ calculations*

From the dose-mortality figures for each compound the $LD_{50}$ was calculated, and the $LD_{50}$ of the anhydrous form of the cobalt compound and the $LD_{50}$ based on the cobalt(II) ion were derived from these data (Table 1). The oral $LD_{50}$ values calculated for the anhydrous cobalt(II) compounds were (in mg/kg body weight): fluoride 150, oxide 202, phosphate 387, bromide 406, chloride 418, sulphate 424, nitrate 434 and acetate 503.

*Histopathology*

Histopathological examination of animals that died during the experimental period was difficult because of autolytic changes. Gross examination of rats given cobalt(II) fluoride revealed severely haemorrhagic mucosal tissue and in addition the stomach was filled with a colourless fluid. Histopathological examination of the liver of the animals that died in the groups treated with cobalt(II) fluoride showed glycogen depletion (Table 2). Frequently a diffuse vacuolization was noted in these livers. In a number of animals hyperaemia was found, particularly in the liver and kidneys. Cytoplasmic changes in the liver were seen at all dose levels except the lowest. The cytoplasm was very granular or clumpy and was located against the cell membrane, giving the impression of an empty cell. Sometimes the cytoplasm was partly eosinophilic. Three of the animals given the 290-mg/kg dose showed myocardial changes consisting of proliferative and oedematous interstitial tissue and swollen muscle fibres with focal degeneration. In the kidneys of all the fluoride-treated animals, the glomeruli were very rich in cells and basal membranes were thickened; cells of the proximal tubules were swollen and exhibited slight vacuolization and degeneration. In four animals of the 290-mg/kg group this degeneration was more pronounced; the apical part of the cells was often very eosinophilic and sometimes contained granular material, the epithelial cells showed degeneration, the microvilli had disappeared and in some cases mitotic activity was observed, while in the lumen, cell debris and foamy material were noted.

Microscopic investigation of animals dosed with cobalt(II) oxide showed changes (Table 3) similar to those in the fluoride-treated animals. In addition to hyperaemia, some haemorrhage was observed, es-

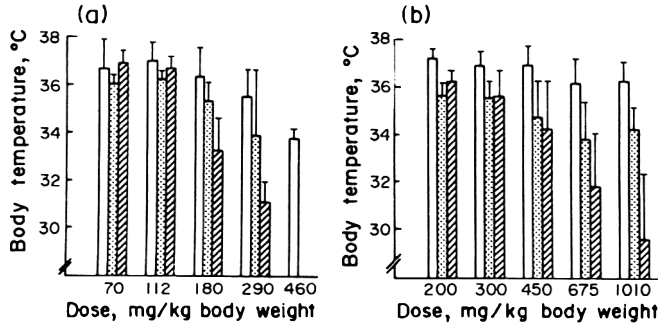


Fig. 1. Body temperatures taken 1·5 (□), 24 (▨) and 48 (▩) hr after administration of a single dose of (a) cobalt(II) fluoride and (b) cobalt(II) oxide to rats. The values are means for groups initially of five male and five female rats, with bars indicating 1 SD.

Table 2. *Histopathological findings in the heart, liver and kidneys of rats treated orally with cobalt(II) fluoride in a single dose of 70–290 mg/kg body weight*

| | Incidence* following a single dose (mg/kg) of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 70 | | 112 | | 180 | | 290 | |
| Finding Sex... | M | F | M | F | M | F | M | F |
| **Heart** | | | | | | | | |
| No. examined... | 5 | 5 | 5 | 5 | 5 | 5 | | 5 |
| Hyperaemia and haemorrhagic changes in myocardium | | | | | 1 | | | 3 |
| **Liver** | | | | | | | | |
| No. examined... | 5 | 5 | 5 | 5 | 5 | 5 | | 5 |
| Diffuse vacuolization | | 1 | 2 | 2 | | | | 5 |
| Glycogen depletion—slight | | | | 1 | | | | 1 |
| —severe | | 1 | | | 3 | 3 | | |
| Hyperaemia | | 1 | 2 | 4 | 2 | 1 | | |
| Focal necrosis | 1 | | | | | | | |
| Cytoplasmic changes | | | 2 | 1 | 2 | 2 | | 2 |
| **Kidneys** | | | | | | | | |
| No. examined... | 5 | 5 | 5 | 5 | 5 | 5 | | 5 |
| Hyperaemia | | 1 | 1 | | 3 | 2 | | 1 |
| Focal interstitial nephritis | 1 | | | | | | | |
| Calcium deposits in cortex and juxtamedullary area | | | | 1 | | | | |
| Swollen proximal tubules | 3 | 1 | 4 | 2 | 3 | 3 | | |
| Slight vacuolization and degeneration of tubules | 1 | | 3 | 1 | 1 | 2 | | |
| Autolytic changes | | | | 1 | 2 | 2 | | 4 |

*No. of organs affected out of the stated no. examined. The organs examined were taken from all the rats surviving 48 hr or more after treatment (initial group sizes, five males and five females).

Table 3. *Histopathological findings in the heart, liver and kidneys of rats treated orally with cobalt(II) oxide in a single dose of 200–1010 mg/kg body weight*

| | Incidence* following a single dose (mg/kg) of | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 200 | | 300 | | 450 | | 675 | | 1010 | |
| Finding Sex... | M | F | M | F | M | F | M | F | M | F |
| **Heart** | | | | | | | | | | |
| No. examined... | 5 | 3 | 3 | 4 | 4 | 2 | 4 | 1 | 4 | 1 |
| Hyperaemia | 1 | | 1 | | | | | | 3 | |
| Haemorrhage | | | 1 | | | | | | 3 | |
| Subendocardial haemorrhage | | | | | | | | | 2 | |
| Interstitial oedema and cell proliferation | | | 1 | | 1 | 1 | 3 | 1 | 3 | |
| Myocardial changes | | | | | 1 | 1 | | 1 | 1 | |
| **Liver** | | | | | | | | | | |
| No. examined... | 5 | 3 | 3 | 4 | 4 | 2 | 4 | 1 | 4 | 1 |
| Periportal cellular infiltrates | 1 | 2 | | | 1 | | | | | |
| Glycogen depletion—moderate | 1 | 1 | | 1 | | | | | 3 | |
| —severe | 1 | 1 | 1 | 1 | 2 | 1 | 4 | 1 | 1 | 1 |
| Vacuolization | 2 | 1 | | 1 | 2 | 2 | 1 | | 2 | 1 |
| Hyperaemia | 3 | 3 | 2 | 3 | 2 | | | | | |
| Enlarged and eosinophilic cells | 1 | | 3 | 2 | 2 | | 1 | 1 | 3 | |
| Necrotic foci | 1 | | 1 | 2 | | | | | | |
| Periportal cells very pale | | | | | | | 2 | | 3 | |
| **Kidneys** | | | | | | | | | | |
| No. examined... | 5 | 3 | 3 | 4 | 4 | 2 | 4 | 1 | 4 | 1 |
| Slight nephritis | | 1 | 1 | | | | | | | |
| Hyperaemia | 5 | 3 | 3 | 3 | 3 | | 3 | | 4 | 1 |
| Haemorrhage | | | | 1 | | | | | 1 | |
| Tubular alterations | 4 | 2 | 3 | 3 | 3 | 1 | 4 | | 4 | 1 |
| Autolytic changes | | | | | | 1 | 2 | 1 | | |

*No. of organs affected out of the stated no. examined. The organs examined were taken from all the rats surviving 48 hr or more after treatment (initial group sizes, five males and five females).

pecially in the heart. Also in the heart, swollen and eosinophilic muscle fibres were observed. In some animals (Table 3) vacuolization, degeneration and necrosis of the myocardium was associated with disappearance of the cross-striations. In these areas mitotic activity was also apparent, probably in developing fibroblasts.

## Discussion

Calculated on the basis of the cobalt ion, the acute oral toxicities of the cobalt compounds tested are similar. Except for the bromide and fluoride compounds, which showed a somewhat higher acute toxicity, the contribution of the cation to the toxicity of the compound is negligible compared with that of the cobalt anion.

The oral $LD_{50}$ of cobalt(II) oxide is considerably lower than that reported by Smyth *et al.* (1969). This difference in acute toxicity could have been due to the different vehicle or to differences in the strain or body weight of the rats. In the present study a suspension of cobalt(II) oxide in 1% carboxymethylcellulose was prepared shortly before administration, whereas Smyth *et al.* (1969) used a 0·25% agar suspension. The strain and body weight of their rats were not reported.

The decrease in body temperature observed in the present study confirms the results of Burke (1978), who found a maximal decrease of 5°C after ip administration of 25 mg cobalt(II) chloride/kg body weight to mice. In this case, however, the temperature was restored to normal after 24 hr. Lowering of the body temperature is possibly due to inhibition of oxidative processes by cobalt. This possibility is supported by the inhibition of glucose oxidation demonstrated after oral dosing of cobalt chloride to mice (Isom & Way, 1974) and the elevation of blood lactate reported to occur in rats both after administration of cobalt and after hypoxia (Rodgers, Fisher & George, 1974). Histopathological observations such as fatty accumulation in the myocardium also indicate inhibition of oxidative processes in the mitochondria (Grice, Goodman, Munro *et al.* 1969; Hall & Smith, 1968).

Long-term administration of cobalt(II) chloride to rats has been found to cause oedematous changes in interstitial tissue, fragmentation and vacuolization in heart-muscle cells, swollen mitochondria with loss of cristae, disappearance of cross striation and myocardial degeneration (Grice *et al.* 1969; Hall & Smith, 1968). In the present acute toxicity study similar effects were observed after a single dose of cobalt(II) fluoride or oxide.

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# ISOFLAVONE CONTENT OF SOYA-BASED LABORATORY ANIMAL DIETS

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**Abstract**—The soya phyto-oestrogens (isoflavones)—genistein, daidzein (and their glucosides, genistin and daidzin) and coumestrol—are reported to contribute to the hypocholesterolaemic response to soya-protein foods. Examination of several soya-based diets for laboratory animals has shown the diets to have a highly variable content of isoflavones.

## Introduction

The soya isoflavones, genistein, daidzein (and their glucosides, genistin and daidzin) and coumestrol have been recognised for some time as the source of the oestrogenic activity demonstrated in some animal diets by the mouse uterine growth bioassay (Bickoff, Livingston, Hendrickson & Booth, 1962). Assays of oestrogen receptor binding have shown that these isoflavones mimic 17-$\beta$-oestradiol, although the binding constants are far less than those of the mammalian oestrogens (Martin, Horwitz, Ryan & McGuire, 1978; Verdeal, Brown, Richardson & Ryan, 1980).

Another reported bioactivity of these isoflavones has been their ability to lower serum cholesterol (Sharma, 1979a,b; Siddiqui & Siddiqui, 1976). In fact, some researchers strongly suggest that the isoflavones in soya are, in part, responsible for the hypocholesterolaemic effect of soya protein compared with animal-protein foods (*Nutrition Reviews*, 1980).

Recently, Drane, Patterson, Roberts & Saba (1980) have reported the extraction of oestrogenically active fractions from various soya-containing laboratory-animal and human foods. We recently reported a method for the quantitative analysis of soya foods for all five forms of the isoflavones (Murphy, 1981) and have now utilized this method to analyse several laboratory-animal diets. We were interested in the isoflavone content of these purified soya-based diets mainly because of their potential use in studies of the ability of plant proteins to lower serum cholesterol. The data show considerable variation in the isoflavone content of these diets.

## Experimental

*Materials.* Soya-based diets for laboratory animals were obtained from several commercial sources. Two (diets 2 and 6) were composed of an enzymatic hydrolysate of soya protein. The other five diets had various protein contents, with little other designation than 'soya protein'. Diets 4 and 5 were from the same supplier and were labelled grades II and I, respectively.

*Analysis.* Protein was determined according to the AOAC method (Association of Official Analytical Chemists, 1980). The soya isoflavones were determined by a high-performance liquid chromatographic method that we developed recently for soya foods (Murphy, 1981).

## Results and Discussion

The diets varied considerably in protein content, as shown in Table 1. Because of this variability, the isoflavone data were expressed in two ways. One was on an "as is" basis, giving the levels in the total diets as received; the other was on a protein basis, so that comparisons between diets could be made. The isoflavone contents of diets 3 and 4 were similar to those of the grade of soya isolates used for human food (Murphy, 1982). The enzymatic hydrolysates (diets 2 and 6) had much lower levels of genistin than did the other diets. We have made similar observations with water-treated soya proteins. When soya beans were germinated, when soya isolate was prepared from analysed varieties of whole soya beans, or when tofu was prepared (by precipitation of water-soluble soya proteins with calcium), the content of the glucoside forms decreased dramatically, but there was no apparent increase in the free forms, genistein and daidzein.

The differences between diets 4 and 5 indicate that the treatment used by the supplier of these diets to change diet 4 (grade II) to diet 5 (grade I) significantly reduced the isoflavone content. This decrease is greater than can be explained by the 23% decrease in protein content.

Coumestrol has been reported to occur as a parts per billion (b = $10^9$) component of whole soya beans (Lookhart, Jones & Finney, 1978) and in parts per million in germinated seeds (Lookhart, Finney & Finney, 1980). No coumestrol was detected at the ppb level in these soya-based diets.

The data in Table 1 have also been calculated in terms of the minimum dietary consumption equivalent to 8 mg genistein. This was the minimum dose reported by Bickoff *et al.* (1962) to give an oestrogenic response in mice. The data are also expressed in Table 1 in diethylstilboestrol (DES) equivalents according to Drane *et al.* (1980) and genistein equivalents according to Bickoff *et al.* (1962). These calculations indicate the maximum response that could be expected if all the isoflavone in the diets were in the form of genistein. Few data are available on the bioactivity of the

Table 1. *Isoflavone levels and oestrogenic dose equivalents of commercial soya-based diets for laboratory animals*

| Diet no. | Analytical data* (concns in ppm) | | | | | | | | Oestrogenic dose equivalents | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Genistin | | Daidzin | | Genistein | | Daidzein | | | |
| | Protein concn (%) | In total diet | On protein basis | In total diet | On protein basis | In total diet | On protein basis | In total diet | On protein basis | Total isoflavone content (ppm genistein equivalents†) | DES equivalent‡ (ppb) | Potentially oestrogenic dietary intake§ (g) |
| 1 | 62·01 | 65 | 105 | 16 | 26 | 16 | 26 | 0 | — | 93 | 0·96 | 86 |
| 2 | 61·60 | 18 | 30 | 14 | 22 | 0 | — | 0 | — | 28 | 0·29 | 283 |
| 3 | 81·56 | 421 | 516 | 73 | 90 | 68 | 83 | 5 | 6 | 546 | 5·66 | 15 |
| 4 | 83·17 | 450 | 542 | 67 | 81 | 68 | 82 | 0 | — | 566 | 5·88 | 14 |
| 5 | 63·64 | 64 | 101 | 18 | 28 | 0 | — | 0 | — | 77 | 0·80 | 103 |
| 6 | 58·31 | 13 | 22 | 0 | — | 11 | 19 | 0 | — | 24 | 0·25 | 332 |
| 7 | 78·81 | 29 | 36 | 0 | — | 15 | 19 | 1 | 1 | 44 | 0·46 | 181 |

*Data are the results of triplicate analyses. Recoveries of added isoflavones averaged 78%.
†Total genistein, genistin, daidzin and daidzein (ppm of whole diet); 11 mg daidzein is equivalent to 8 mg genistein.
‡Oestrogenic activity of 8 mg genistein is equivalent to that of 0·083 μg DES.
§Weight of diet provided 8 mg genistein, the minimum dose reported by Bickoff *et al.* (1962) to induce an oestrogenic response in mice.

glucosides, genistin and daidzin, although these forms account for 95–99% of the isoflavone content of whole soya beans (Murphy, 1982; Naim, Gestetner, Zilka *et al.* 1974). Therefore, the data in Table 1 are only a rough estimate of the oestrogenic activity of these diets.

It is not possible at this time to estimate a potential hypocholesterolaemic response to the isoflavones found in soya-based diets. Hypocholesterolaemic activities have been measured in rats for several isoflavones found in lesser-known non-American varieties of legumes (Sharma, 1979a). Daidzein was the only isoflavone found in both these legumes and soya beans. Sharma (1979a) concluded, however, that daidzein had no hypocholesterolaemic activity in rats because not all legumes containing daidzein were able to lower serum cholesterol, The report did not take into account β-sitosterol or the other saponins that would be expected in these legumes and that might be expected to contribute to the hypocholesterolaemic response (Birk & Peri, 1980). Moreover, it is known that the isoflavones are metabolized to different end products in different species (Verdeal & Ryan, 1979). Therefore, it is probable that they elicit different hypocholesterolaemic responses in different species.

Because there is great interest in the cholesterol-lowering ability of soya and other plant-protein foods, we believe it is important that researchers in this area be made aware of the non-protein components of their soya-based diets and the potential effect of these compounds on the interpretation of their data. Much work remains to be done to determine what role, if any, the soya isoflavones play in the hypocholesterolaemic activity of soya-bean protein.

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# Review Section

## SPECULATIONS ON AN EXTENDED DOSE-RESPONSE MODEL FOR CARCINOGENESIS

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**Summary**—As an alternative method of model selection for low-dose risk assessment, it is possible to evaluate a particular mathematical dose-response model with respect to several dimensions of the overall carcinogenic process. An extended form of the Weibull model is proposed for further evaluation.

### Introduction

One method of estimating the risk of cancer at a very low exposure from the experimental results with laboratory animals is to fit a dose-response function to the observed results at high doses and to extrapolate to the low dose of interest with the fitted function. Several mathematical models have been proposed for this procedure. These models, however, can produce very different low-risk assessments from the same data set. Until recently, it was hoped that the results from very large experiments with laboratory animals would solve the problem of model selection and other related problems. It is now clear that this was a false hope. The results from even the largest experiment can usually be fitted in the statistical sense by many models. Even the question of whether there is a threshold or not is unresolved by these experimental results.

The problem of low-risk assessment thus persists. Another approach to evaluating a mathematical model for low-risk assessment, is to test it against the experimental results from cancer research that is not directly related to low-risk assessment. The assumption here is that a simple, reasonable model that fits the data over a broad region of observable results is also a good model for extrapolating to the unobservable region of low-risk assessment. This is, of course, the traditional method of science. For example, it was not necessary to visit the site to determine the gravitational effect of the moon on a human visitor. This effect was calculated in advance by a mathematical model developed over previously explored experimental regions. For the purposes of this discussion, it is also important to observe that only a model was required, not an understanding of the true mechanism of gravity.

The Weibull model has been proposed for low-risk assessment (Carlborg, 1981a,b,c & 1982). In the spirit of the preceding paragraph, this article offers extended versions of the Weibull model which can be tested over a broad experimental region not generally associated with the problem of low-risk assessment. The exposition is highly speculative, and only selected experimental results are given to illustrate the concepts.

### The problem

The situation of interest can be described in three ways: in words, in a picture and in mathematical symbols. Figure 1 gives a picture of the situation. There are three axes, one for each of three dimensions to the overall problem. The goal for low-risk assessment is to determine the risk at point A, which represents an animal exposed for a long time (axis 1) at a low dose (axis 2) exhibiting a severe endpoint (a tumour, say) in the carcinogenic process (axis 3). Experimentally, this is an unexplorable region. A typical dose-response experiment is indicated by point B, which represents animals exposed for a long time (axis 1) at high doses (axis 2) exhibiting tumours (axis 3). A dose-response model for low-risk assessment is an attempt to go across the doses from point B to point A. As mentioned above in the Introduction, very large recent experiments may be viewed as an unsuccessful attempt at model selection. On the other hand, a relatively small experiment at a very few fortuitously

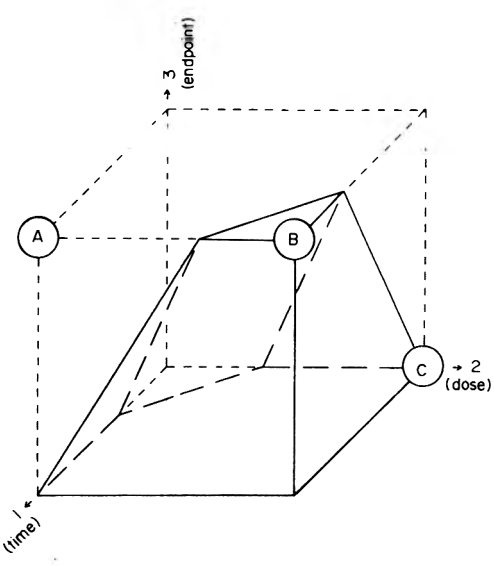


Fig. 1. Heuristic representation of the three dimensions to the carcinogenic process and the experimentally observable region.

chosen doses would be sufficient for any particular selected model.

The large cube in Fig. 1 is supposed to indicate the conceivable boundaries to the overall situation in terms of these three dimensions (time, dose and endpoint). There is a region of this cube defined by the solid lines (and the hidden long-dashed lines). Crudely, this is supposed to indicate the observable region. For example, consider an experiment represented by point C. This would be of short duration (axis 1), at high doses (axis 2) and exhibiting a mild endpoint (axis 3) of the carcinogenic process. Excess mitotic activity might be an example of such a mild endpoint. It is implied in the construction of Fig. 1 that less severe endpoints can be observed in shorter times and at lower doses.

The main idea of this article is that a mathematical model should be sought for the entire observable region suggested by Fig. 1. This model would then be used for low-risk assessment (going to point A). It is conceivable that ultimately a carcinogen could be evaluated solely by an experiment represented by point C. This would be a short-term experiment. In particular, extensions of the Weibull model will be offered to meet some of the problems expected in building a global model for the experimentally observable region.

## The Weibull model

In its simplest form for dose-response situations, the Weibull model is

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

where P is the probability of a response (tumour, say), d is the dose, and m, $\alpha$ and $\beta$ are parameters to be estimated from the data. In terms of the background probability of a response ($P_0$), this may be written as

$$P = P_0 + (1 - P_0)(1 - e^{-\beta d^m}).$$

Expressed as the risk over the background, it is

$$P = 1 - e^{-\beta d^m}, \qquad \text{(W1)}$$

where the 'P' on the left side is now the excess risk ($=(P - P_0)/(1 - P_0)$). To simplify the discussion, take model W1 as the basic Weibull model and ignore the background response. This is then the Weibull model for going from point B to point A in Fig. 1.

The goal of this section is to suggest extended forms of the basic Weibull model—that is, models for moving away from point B in Fig. 1 to the other parts of the observable region. First consider moving backwards on the time axis. Such an experiment would include early kills (less than the lifetime of the test animal). In theory, both parameters ($\beta$ and m) might depend on the time of the kill giving

$$P = 1 - e^{-\beta(t) d^{m(t)}}, \qquad \text{(W2)}$$

where $\beta(t)$ and m(t) are functions of the time t. In the persistent interest of a more simple model, consider

$$P = 1 - e^{-\beta(t) d^m}, \qquad \text{(W3)}$$

where the shape parameter m for the dose does not depend on the time of the kill. As an even simpler model, consider

$$P = 1 - e^{-\beta t^k d^m}, \qquad \text{(W4)}$$

where now there is a shape parameter k for the time of the kill and a constant value of the parameter $\beta$ for all doses and times. Model W4 has been successfully fitted to the results for the liver for mice exposed by ingestion to 2-AAF (Carlborg, 1981c). Since this experiment provides the largest data set available for testing W4, this is an encouraging outcome.

Next consider moving downward from point B of Fig. 1 to the less severe endpoints. Initially, take only lifetime experiments (t held constant). An extension of the basic Weibull model W1 to include this dimension is

$$P = 1 - e^{-\beta(s) d^{m(s)}}, \qquad \text{(W5)}$$

where s is the endpoint (or stage). In model W5, $\beta(s)$ and m(s) are functions of this endpoint. The endpoint is not quantitatively measured like the dose d or the time t. It takes on a discrete value like 'carcinoma', 'adenoma', 'hyperplasia' or 'mitosis'. The next two sections are devoted to discussions of hypotheses concerning model W5, especially with reference to the dependence of the shape parameter m on the endpoint.

Finally, consider moving away from the point B of Fig. 1 in any direction to any position in the observable region. A model for this region would be the desired global model. The natural extension of the Weibull model is

$$P = 1 - e^{-\beta(s) t^{k(s)} d^{m(s)}}, \qquad \text{(W6)}$$

where k(s) is a function of the endpoint s and the other symbols are defined above.

One extremely speculative simplification of model W5 can be offered. This is related to the work of Druckrey (1967) and his colleagues. When this work is reconsidered from the viewpoint of the Weibull model, it appears that the shape parameter for the time to a tumour (Druckrey's endpoint) and the shape parameter for the time to a kill are the same thing. If so, then the exponent in model W6 may be rewritten as

$$\beta(s)(dt^{n(s)})^{m(s)},$$

where $n(s)(=k(s)/m(s))$ is Druckrey's number. Now boldly assume that Druckrey's number is constant across endpoints. Then, the extremely speculative model is

$$P = 1 - e^{-\beta(s)(dt^n)^{m(s)}}, \qquad \text{(W7)}$$

where n is a constant not depending on the endpoint.

## Relationship between shape parameter and severity of endpoint

This section and the next discuss two hypotheses concerned with the problem of building a model across the endpoints. Mathematically, these hypotheses deal with the function m(s) (see model W5), which relates the value of the shape parameter m for the dose to the endpoint s.

The best experimental results for illustrating the main idea of this section are those from a study with ethylene thiourea given in the diet to rats. Thyroid tumours were produced as shown in Fig. 2. To illustrate, consider the vertical bar for the group exposed at 250 ppm. About 85% of the rats developed hyper-

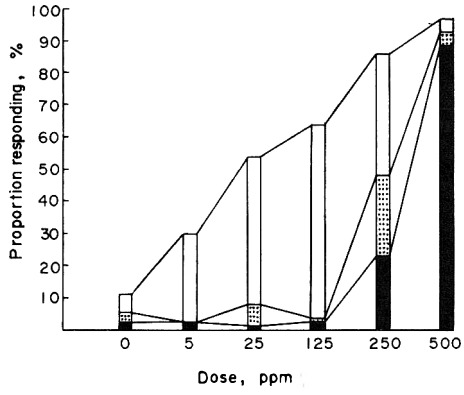


Fig. 2. Thyroid responses, hyperplasia (□), adenoma (▦) and carcinoma (■), of rats exposed to ethylene thiourea in the diet (data from Graham, Davis, Hansen & Graham, 1975).

plasia, an adenoma or a carcinoma of the thyroid, about 45% developed an adenoma or a carcinoma and about 25% developed a carcinoma. Even with this crude bar chart, one can see the variation among the three dose-response curves. With hyperplasia or worse as the endpoint, the curve falls off very slowly as the doses are decreased. With a carcinoma as the endpoint, the curve falls off most sharply.

Through the Weibull model, the qualitative observations of the preceding paragraph can be quantified. Table 1 gives the observed data from the experiment with ethylene thiourea and the results of fitting the Weibull model to the three different endpoints. The important conclusion is that as the severity of the endpoint increases (hyperplasia to adenoma to carcinoma) so does the value of the shape parameter m (0·51 to 2·63 to 3·31). (The experimenters mention finding different grades of hyperplasia, but only the data for overall hyperplasia are reported. One wonders if an intermediate grade of hyperplasia would produce a value of m near the missing value of about 1·5.)

The general pattern just described has occurred frequently and consistently in the author's experience with reported data sets, giving the motivation for the hypothesis of this section: within a given experiment, the value of the shape parameter m for the dose is an increasing function of the severity of the endpoint s. In other words, a more severe endpoint has a steeper dose-response curve (larger value of m) than a less severe endpoint. Unfortunately, no explicit form of the function can be offered at this time. For expository purposes only, one might consider

$$m_i = m_{i-1} + c,$$

where i − 1 and i refer to two successive endpoints and c is a positive constant (perhaps depending on i).

Another illustration of this hypothesis is the outcome of the large experiment with mice exposed by ingestion to 2-AAF, where the bladder was one of the target organs. Hyperplasia and a neoplasm are the two endpoints. Table 2 gives the reported data and the results of fitting the Weibull model to these data. With hyperplasia or worse as the endpoint, the estimated value of m is 5·75. With a neoplasm as the endpoint, the estimated value of m is 6·35.

As a slight digression, Table 2 offers another test of model W3, which says that the value of the shape parameter for the dose is constant across the kill times. Notice that with a bladder neoplasm as the endpoint the model fits with a single value of the shape parameter (m = 6·35). Other kill times produced relatively meagre responses not offering a real test. (The data for hyperplasia or worse as the endpoint at 18 months have not been obtained from the National Center for Toxicological Research.)

## Non-dichotomous endpoints

The second problem encountered in building a model across the endpoints (moving downward from point B of Fig. 1) is that the less severe endpoints are naturally measured differently from the more severe endpoints. The Weibull model, as so far discussed, is applicable to the dichotomous severe endpoints only. That is, an animal either has the tumour of interest, or it does not have the tumour. The process of carcinogenesis involves endpoints (stages) that are not dichotomously measured. Excessive mitosis in the target organ is an example, where the response is measured by the level of mitotic activity—not its presence or absence. This section offers an alternative

Table 1. *Reported pathology of the thyroid for rats exposed to ethylene thiourea and the results of fitting the Weibull model to these data**

| Pathological endpoint | Dose... / No. at risk... | 0 / 72 | 5 / 75 | 25 / 73 | 125 / 73 | 250 / 69 | 500 / 70 | P | Est. m | SE m |
|---|---|---|---|---|---|---|---|---|---|---|
| Hyperplasia, adenoma or carcinoma | Observed†‡... | 8 | 22 | 44 | 47 | 59 | 68 | | | |
| | Calculated§... | 8·2 | 23·8 | 37·3 | 56·2 | 60·0 | 66·0 | 0·03 | 0·51 | 0·062 |
| Adenoma or carcinoma | Observed†‡... | 4 | 2 | 6 | 3 | 33 | 65 | | | |
| | Calculated§... | 3·4 | 3·6 | 3·5 | 8·5 | 27·7 | 66·2 | 0·04 | 2·63 | 0·32 |
| Carcinoma | Observed†... | 2 | 2 | 1 | 2 | 16 | 62 | | | |
| | Calculated§... | 1·5 | 1·6 | 1·5 | 3·1 | 14·8 | 62·2 | 0·77 | 3·31 | 0·42 |

Est. m = The statistical best estimate of the shape parameter in the Weibull model
SE m = The approximate standard error for the best estimate of m

*Data were obtained from Graham, Davis, Hansen & Graham (1975) and the model was fitted by the method of iterated weighted least squares.

†Observed no. of animals affected.

‡These data are approximate, having been read from a graph in Graham *et al.* (1975).

§Calculated no. of animals affected (Weibull model).

Table 2. *Reported pathology of the bladder for mice exposed to 2-AAF and the results of fitting the Weibull model to these data**

| Pathological endpoint | Kill time (months) | Dose (ppm)... | 0 | 30 | 35 | 45 | 60 | 75 | 100 | 150 | P | Est. m |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hyperplasia or neoplasm | 24 | No. at risk... | 384 | 900 | 638 | 445 | 415 | 311 | 160 | 130 | | |
| | | Observed†‡... | 3 | 16 | 16 | 12 | 30 | 69 | 113 | 126 | | |
| | | Calculated§... | 6·2 | 15·5 | 12·1 | 12·5 | 32·0 | 68·0 | 112·9 | 130·0 | 0·70 | 5·75 |
| Neoplasm | 18 | No. at risk | 400 | 1573 | 796 | 383 | 269 | 267 | 131 | 121 | | |
| | | Observed†... | 1 | 4 | 1 | 1 | 3 | 1 | 5 | 62 | | |
| | | Calculated§... | 0·9 | 3·7 | 1·9 | 1·0 | 1·2 | 2·8 | 7·0 | 60·2 | | |
| Neoplasm | 24 | No. at risk... | 384 | 900 | 638 | 445 | 415 | 311 | 160 | 130 | | |
| | | Observed†... | 1 | 0 | 2 | 1 | 3 | 3 | 25 | 100 | | |
| | | Calculated§... | 0·6 | 1·4 | 1·0 | 1·0 | 2·5 | 6·3 | 18·1 | 102·5 | 0·29 | 6·35 |

Est. m = The statistical best estimate of the shape parameter in the Weibull model

*Data were obtained from Staffa & Mehlman (1979) and the model was fitted by the method of iterated weighted least squares.

†Observed no. of animals affected.

‡These data were kindly supplied by Charles Frith of the National Center for Toxicological Research.

§Calculated no. of animals affected (Weibull model).

form of the Weibull model which can be used for both types of endpoints in a consistent manner.

To motivate the derivation, consider a tumour as the (dichotomous) endpoint. For a particular dose group of (say) 50 animals, suppose there are 75 tumours in the organ of interest or an average of 1·5 tumours per animal. Tentatively, assume that these 75 tumours are distributed randomly (Poisson distribution) among the 50 animals. Then the expected proportion of animals having at least one tumour is $1 - e^{-1.5} = 0.78$, which can be compared to the observed proportion of tumour-bearing animals. Now suppose that in a large number of such comparisons the expected and observed proportions of tumour-bearing animals agree very well in the statistical sense. Then one would conclude that the assumed random distribution of tumours among the animals was valid. This would have interesting implications for the Weibull model. To see this, let Y be the average number of tumours per animal in a dose group. Under the Poisson distribution, the probability P of a tumour-bearing animal is

$$P = 1 - e^{-Y}.$$

Under the Weibull model, this probability for a dose d is

$$P = 1 - e^{-\beta d^m}.$$

Therefore,

$$P = 1 - e^{-Y} = 1 - e^{-\beta d^m}.$$

Thus, the desired model is

$$Y = \beta d^m, \qquad \text{(W1B)}$$

or

$$\log_e Y = \log_e \beta + m \log_e d. \qquad \text{(W1C)}$$

This is the alternative form of the model W1, where the endpoint is no longer measured dichotomously. But the parameter m is still the shape parameter with its previous interpretation. Clearly, this form of the Weibull model can then be applied to a non-dichotomous endpoint like mitotic activity.

The critical step in the preceding argument is that the tumours are distributed randomly among the animals. This needs experimental justification. Essential to such a justification is the need to count all the tumours in the organ of interest, rather than just the presence or absence of a tumour in the organ. The results from one very large study are relevant. Mice were injected ip with 18 different alkylating agents producing dose-related lung tumours. In all, there were 79 different dose groups including the controls. For each group, the average number Y of tumours per animal was meticulously measured, as well as the proportion P of tumour-bearing animals. Figure 3 gives a picture of the results. The horizontal axis is for the calculated proportion of tumour-bearing animals ($1 - e^{-Y}$), and the vertical axis is for the observed proportion of tumour-bearing animals (P). Each small circle represents a result from one of the 79 experi-

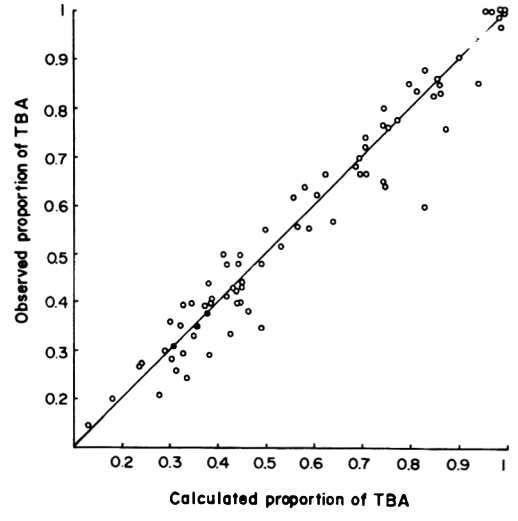


Fig. 3. A comparison of the calculated and observed proportions of tumour-bearing animals (TBA) for mice developing lung tumours in response to various alkylating agents (data from Shimkin, Weisburger, Weisburger *et al.* 1966). The calculated proportion of TBA was derived from the average number of tumours per animal. Treated groups (○) comprised 12 to 54 mice and control groups (●) comprised 108, 330 or 339 mice.

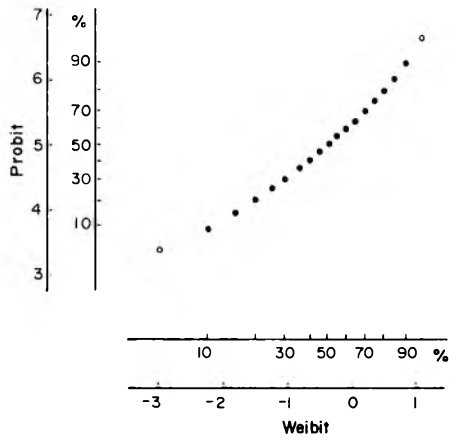


Fig. 4. The relationship between probit and Weibit. A response in the 10 to 90% range (●); a response of 5 or 95% (○).

mental groups. These circles line up extremely well along the 45-degree line corresponding to the equivalence of the observed and calculated proportions of tumour-bearing animals ($P = 1 - e^{-Y}$). This is one example of the desired experimental justification. Certainly, further justifications are necessary under different experimental conditions (strains, species, carcinogens, routes of exposure, target organs etc.).

Using the procedure just illustrated, one can easily derive the alternative form(s) of each Weibull model discussed earlier.

**The relationship between the Weibull and probit models**

Many experimental toxicologists have a strong attraction to the probit model for bioassays. There seem to be two main reasons for this attraction. (1) The probit model has served well for a long time; for example in the determination of the $LD_{50}$. (2) Biological responses are believed to be inherently related to the logarithm of the dose.

For such an experimenter, it is as well to observe that the Weibull model offers nothing that is new or different. A probit scale for the response is actually no more than a mathematical device which renders the response on a vertical axis linear relative to the logarithm of the dose on a horizontal axis. As indicated by model W1C, the Weibull model does the same thing with

$$\log_e Y = \log_e(-\log_e(1 - P)) = W \text{ (say)}$$

on the vertical axis. Call the new scale a Weibit scale (W). Figure 4 shows the general relationship between the Weibit (horizontal axis) and the probit (vertical axis). In the range of interest for most bioassays (the 10 to 90% responses), the relationship between the scales is essentially linear. This implies that data that appear linear on a probit graph would also appear linear on a Weibit graph. Furthermore, an $LD_{50}$ calculated one way would be essentially the same as an $LD_{50}$ calculated the other way.

The important difference between the two models is in the extremes, and low-risk assessment is a problem at one extreme. The Weibull model is more conservative (higher risk), with the difference between the two increasing rapidly at lower doses.

*Acknowledgements*—I thank Leon Golberg, Stan Vesselinovitch and Virgil Wodicka for their helpful comments on this and related projects.

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# REVIEWS OF RECENT PUBLICATIONS

**Handbook of International Food Regulatory Toxicology. Volume 1: Evaluations; Volume 2: Profiles.** Edited by G. Vettorazzi, MTP Press Ltd, Lancaster, 1980. Vol. 1, pp. 161, £14.95; Vol. 2, pp. 191, £14.95.

The growing awareness of the need for consistent testing methods and for detailed evaluation of the food additives that are intentionally or unintentionally included in our foods has led to the establishment of a number of expert panels who have been prolific in their publications. Their comments and recommendations have been widely acknowledged and have contributed to many current practices. However, these comments have been spread over many documents and the present two volumes attempt to collect these statements together to form an integrated reference work and also to summarize some of the main findings on one of the major classes of food additives—food colourings.

In volume 1, the first chapter concentrates on the work of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and begins by detailing the principles that it uses to assess the safety of a compound. It is stressed that JECFA considers it important to balance risk and benefit and to ensure that well-tested materials are not replaced by ones with few toxicological studies behind them. An excellent section on the considerations to be taken into account in toxicological testing procedures follows. The rationale behind the advised procedures is clearly outlined and the importance of careful statistical analysis is emphasized. There then follows a discussion, in some detail, of the interpretation and evaluation of toxicological data which provides much information that would be of great benefit both to the beginner in toxicology and to those who wish to understand the reasonings behind the official announcements of the various toxicological and legislative panels.

The second chapter of the book deals in a similar way with the work of the Joint FAO/WHO Meeting on Pesticide Residues, outlining the principles, methods, interpretation and evaluation of toxicological testing of pesticides to assess acceptable levels of residues in the human diet. There is a section on the use of the acceptable daily intake values and another on the criteria for toxicological evaluation of some representative classes of pesticide chemicals, covering the organohalogenated compounds, the organomercurials and the organophosphates.

The second volume is somewhat different from the first in that it concentrates on food colourings and attempts to summarize the present toxicological profiles of individual colourings. It is unfortunate, since this is certain to be a book that will be referred to frequently, that the standards of printing are not as high as in Volume 1. However, this does not detract from the quality of the summaries of data which are taken from almost 1200 references and cover more than 100 compounds. A useful table is provided that indicates the data that are available from each of the references and shows up the gaps in our knowledge at a glance.

It is not stated whether these two volumes will become part of a larger series covering other food additives, direct or indirect, or even other industrial or agrochemical compounds but it is certain that any future volumes in a similar style would be welcome. These books are useful additions to the library shelf.

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

This supplement to the journal "Vår Föda" presents the lectures given at a 2-day conference held in Stockholm in February 1981. The aim of the conference was to report and discuss data that could be relevant to a group of Swedish medical experts about to undertake a thorough review of the scientific basis for the continuation of a campaign, begun in the early 1970s, for promoting beneficial food and exercise habits in Sweden. However, although the conference was aimed at generating data for a national health education programme it was attended by an international group of experts who addressed themselves to issues of world-wide importance concerning dietary factors in carcinogenesis.

The first day of the conference was devoted mainly to consideration of specific dietary components and contaminants that may increase the risk of, or protect against, development of various cancers. These included food additives, sweeteners, mycotoxins, *N*-nitrosamines, pesticides, products of pyrolysis, and naturally occurring mutagens, such as certain flavonoids, pyrrolizidine alkaloids, cycasin and safrole. At the end of each published paper is a brief report of the discussion that followed. The conference continued with presentations on clinical aspects of nutrition and cancer, more general papers and a panel discussion.

Although the viewpoints of the contributors were diverse, a general consensus appeared to be that it was advisable to reduce fat and salt intake and to increase the amounts of fibre and fresh vegetables in the diet. Some speakers felt that the levels of intake of certain vitamins could be important. Although this publication contains much information that is not new, it does bring together the views of many experts in the field. The inclusion of an index, presumably excluded to facilitate rapid publication, would have been useful.

**Nickel Toxicology.** Edited by S. S. Brown & F. W. Sunderman, Jr. Academic Press, Inc. (London) Ltd, London, 1980. pp. xx + 193. £15.00.

This compact book is composed of synopses of the scientific papers presented at the Second International

Conference on Nickel Toxicology held in Swansea in September 1980. The steady growth of interest and research into the toxicity of this metal and its compounds, emphasized in the foreword, is clearly demonstrated by the varied and wide-ranging topics considered.

In his opening address Sir Richard Doll debates the relevance of the epidemiological approach to policies for the prevention of cancer and concludes that whilst this is certainly not the investigative method of choice in carcinogenesis, it has a valuable role particularly when conducted in conjunction with laboratory testing. Brief reports of recent epidemiological studies in nickel workers (in whom excess risks of developing cancer, particularly of the respiratory system, have already been identified) are then given. The second section of the book details the advances that have been made in experimental approaches to nickel carcinogenesis. One synopsis reports the potential value of intraocular injection as a procedure for testing the carcinogenicity of nickel compounds. The main advantages of this procedure are a short latent period, a high tumour incidence and the ease of tumour detection.

The salient points of recent *in vivo* and *in vitro* investigations into the uptake, distribution and excretion of nickel compounds are discussed and studies of their toxicity, including genotoxicity, teratogenicity/embryotoxicity, effects on host defences, erythropoiesis, lung clearance and vasoconstriction, and their ability to induce allergic contact dermatitis, are reported.

The final section then critically examines the application of analytical procedures in this field. Among other subjects discussed are procedures for the *in situ* microanalysis of inorganic particles in very small samples of tissue taken from workers' lungs, the *post-mortem* analysis of lung tissue, the detection of nickel in the chromatin of dinoflagellates by X-ray microanalysis and the performance of nickel analyses at trace and ultratrace levels. In addition, interlaboratory comparisons of the results of serum and urine determinations using the IUPAC (International Union of Pure and Applied Chemistry) reference method for nickel are considered.

This book achieves the editors' prime objective of rapid communication of recent research developments. However, since background information has been kept to a minimum and no effort has been made to summarize or explain the impact or implications of the findings as a whole, its effective use by the uninitiated might prove difficult. Nevertheless, each chapter is concisely written and clearly presented and therefore the book provides a convenient update which will represent a welcome addition to the bookshelves of those already familiar with the biological profile of nickel.

**Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment.** Vol. 76. Edited by F. A. Gunther. Springer-Verlag, New York, 1980. pp. viii + 218. DM 54.00.

**Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment.** Vol. 77. Edited by F. A. Gunther. Springer-Verlag, New York, 1981. pp. viii + 364. DM 67.00.

Half of the first volume cited here is occupied with papers contributed by the US delegates to a joint US–Taiwan seminar on "Environmental Problems Associated with Pesticide Usage in the Intensive Agriculture System" held in Taipei in 1979. Topics considered in these contributions include the limitations and advantages of using bioassays for monitoring pesticide residues, the significance and management of insecticide resistance, the interpretation of analytical data on pesticide residues and the effects of pesticides on non-target organisms such as beneficial arthropods, soil micro-organisms and plants, as well as domestic and wild animals.

Possible effects of non-target organisms, including man, also feature in one of the three 'independent' contributions to this volume. This paper considers the use of micro-organisms as insecticides, pointing out that while some successes have been scored with certain viruses and bacteria, the fungi and protozoa studies have tended to be less advantageous replacements for chemical insecticides, since they lack the specificity and rapid action of the more successful micro-organisms. Nevertheless if the insecticidal use of natural components of the environment can indeed sometimes be considered as correcting an ecological imbalance rather than creating a new one, further studies along these lines may well be justified.

Of the two remaining contributions, the first surveys the environmental chemistry of primary aromatic amines and related nitro and azo compounds, dealing systematically with the wide range of reactions in which these compounds are involved and concentrating particularly on those of most importance at the low concentrations generally encountered environmentally. A final section in this paper considers the basic reactions involved in aromatic amine metabolism in higher organisms, particularly mammals. Metabolism is the sole theme of the following contribution in Volume 76, a paper describing the different conjugation systems that enable higher animals to deal with a wide range of foreign chemicals.

In contrast, Volume 77 is concerned exclusively with a pair of closely related herbicides—linuron and monolinuron. This text, by H. Maier-Bode and K. Härtel and generally very adequately translated by H. Behr, describes first the synthesis, physical and chemical properties, herbicidal activity and range of uses of these two substituted methoxymethylureas. A roughly equal number of pages is then devoted to the effects of the herbicides and their formulations on birds and mammals, their behaviour in soils and surface waters and the occurrence and monitoring of residues in agricultural products. An appendix lists relevant registrations in various countries, and the range and depth of this review is indicated by the presence of some 60 pages of references. Those seeking information on these compounds will surely find this volume a profitable starting point for their search.

**Bitter Harvest.** By J. Egginton. Secker and Warburg, London, 1980. pp. 351. £9.95.

This is a tale of the real tragedy that struck farming folk in the North American state of Michigan, the

toxicological ramifications of which may not yet be fully realized. At the time, the events documented in this book went largely unreported in the press, but Joyce Egginton has painstakingly pieced together the story of how thousands of people were exposed to polybrominated biphenyls (PBBs) as contaminants of their food supply, and how these chemicals still persist in the environment.

In the autumn of 1973 whole herds of dairy cattle in Michigan succumbed to a mysterious illness that caused them to lose their appetites, and to become thin and weak; some developed body sores and some died. Milk production virtually ceased, calves were aborted and many animals became sterile. The explanation was found only after months of persistent investigation by a few determined individuals. Cattle feed had become contaminated with PBBs, which were the ingredients of a fire retardant with the trademark Firemaster. The fire retardant was accidentally shipped to a farm co-operative where it was incorporated into dairy feed in place of magnesium oxide, which had been given the trade-name Nutrimaster. The feed-mixing procedures used at the farm co-operative led to cross contamination of other feeds so that livestock such as sheep, pigs and hens were also affected. The authorities were slow to react but eventually contaminated farms were quarantined and some 30,000 animals (approximately 25,000 cattle, 4000 pigs and 1000 sheep, goats and horses) were destroyed and buried at one site alone and about 1·5 million chickens were buried in landfills in various parts of the state.

The problem was not confined to livestock. For over a year, before the accident was discovered and also subsequently while the authorities debated what action to take, people in Michigan were consuming milk, meat and eggs that contained significant amounts of PBBs. During the winter of 1974/75 the farming families, who had consumed the largest quantities of the PBBs in their own produce, began to develop symptoms such as headache, joint pain, fatigue, debilitation, abdominal pains, gastro-intestinal upsets, body sores that would not heal and unaccountable bleeding from the gums and nose. At first, these signs of illness were attributed to the stress that inevitably accompanied prospects of financial ruin. Later, a survey showed that Michigan farming families had an unusually high incidence of physical and neurological ailments and less ability to combat infections than their Wisconsin counterparts. The lack of resistance to infection was related to disturbances of T-lymphocyte distribution and function. Another study found that PBBs were detectable in 96% of the breast milk samples from 108 women who gave birth in Michigan hospitals during August and September 1976. Levels of up to 1·22 ppm were found in the breast milk.

Very little is known about the effects of PBBs on the human body, except that they accumulate in the tissues and are suspected of causing cancer. The symptoms observed in the Michigan citizens were diverse and of a vague nature. The PBBs from the contaminated animal feed have now escaped into the environment and their long-term effects may not be seen for many years. This detailed and graphic account of the disaster is disturbing, not only for what it shows could happen in the first place, but also because of the way the situation was handled and the implications for the future. It was a matter of chance that the feed contaminants were eventually identified as PBBs; if the men who had committed themselves to proving that their cattle were being poisoned had been given ready access to the appropriate expert help and analytical equipment, then the accident might have been discovered a lot earlier.

Unfortunately, some serious-minded readers may be deterred by the author's homely style of writing (the narrative is laced with several examples of mild American expletives reported to have been uttered by her human sources!) which occasionally reads more like a piece of fiction than an accurate scientific account. Nevertheless, the indexing and listing of references and official records is meticulous.

**Banbury Report 4. Cancer Incidence in Defined Populations.** Edited by J. Cairns, J. L. Lyon & M. Skolnick. Cold Spring Harbor Laboratory, New York, 1980. pp. xi + 458. $45.00.

The estimation that environmental factors (including life-style and cultural factors) may account for eighty per cent or more of human cancers has led to increasingly detailed epidemiological investigations in an attempt to identify and assess risk factors. Present evidence strongly suggests that diet, smoking, alcohol and sexual practices markedly influence the incidence of cancer in human populations. However it has been notoriously difficult to partition risk factors, which may themselves be closely associated, in populations that are ill-defined.

The study of defined population is therefore extremely useful and to this end groups of people isolated on geographical, religious or other grounds have proved profitable subjects for research. This book, in the usual format of the 'Banbury Series', presents the papers given at a conference to discuss current work in the field of cancer epidemiology in defined populations. It is divided into three sections dealing with cancer studies in defined populations, genetic studies in defined populations and national death registries.

The first section deals largely with studies conducted on mormons and seventh day adventists, together with epidemiological methodology and risk assessment. The papers cover dietary and social aspects of the two groups and methods of data collection. The papers in the next section describe epidemiological methods for investigating genetic predisposition to disease processes; while largely concerned with cancer, familial associations in coronary heart disease, birth defects and blood pressure are discussed. The difficulties in such studies are probably best exemplified by the Laredo epidemiology project. Detailed analysis of the population in Laredo, Texas is being made in an attempt to correlate genealogies with medical records and to look for risk-clustering for the major degenerative diseases, particulary cancer. Difficulties arise in determining the model for associations, the collection of correct and sufficient data and the analysis of the data. The papers in the final section detail the method of collection of data contained in central registries in the USA and UK

and discuss the suitability of the material collected for epidemiological studies.

The aim of the conference was for experts in the field of cancer epidemiology to present and discuss problems of methodology and interpretation. However, much of the data presented at the meeting are available in the literature, and so the value of publishing the proceedings must be to bring the discussion to a wider audience. In this respect it is a pity that much of the earlier part of the volume is repetitive while in other areas greater detail is required to justify the methods used. In the discussion on the influence of radiation on childhood leukaemias, one author presents data suggesting an increase in leukaemia amongst children in Utah exposed to atomic fallout as a consequence of testing carried out in Nevada in the 1950s, but a second paper severely criticizes the presentation of the data and conclusions drawn. The two papers demonstrate the acrimonious nature of the debate in the USA often exacerbated by considerations not purely of a scientific nature.

An understanding of the power and limitations of epidemiological methods by those not directly involved in the field is to be encouraged. This book is well presented and provides much information, although the reporting of conference proceedings is perhaps not the ideal way of dealing with this topic for a general audience.

## BOOKS RECEIVED FOR REVIEW

**X-Ray Microanalysis in Biology.** Edited by M. A. Hayat. Macmillan Publishers Ltd, London, 1981. pp. viii + 488. £30.00.

**Infections in the Immunocompromised Host—Pathogenesis, Prevention and Therapy.** Edited by J. Verhoef, P. K. Peterson & P. G. Quie. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. viii + 316. Dfl. 80.00.

**Mitogenic Lymphocyte Transformation.** Edited by D. A. Hume & M. J. Weidmann. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. xii + 251. Dfl. 122.00.

**IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.** Vol. 25. **Wood, Leather and Some Associated Industries.** International Agency for Research on Cancer, Lyon, 1981. pp. 412. Sw.fr. 60.00.

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# *Information Section*

## ARTICLES OF GENERAL INTEREST

### TUMOUR PROMOTION BY BHT

One of the more puzzling manifestations of butylated hydroxytoluene (BHT) toxicity in the mouse is lung damage, characterized by hyperplasia, hypertrophy and general disorganization of the cellular components. In DBA/2 mice these effects were minimal after a single ip injection of 40 mg/kg, but prominent at 400 mg/kg (*Cited in F.C.T.* 1973, **11,** 328). The cytodynamic and biochemical nature of the lung changes has been investigated in some detail, using the Swiss–Webster mouse as a model (*ibid* 1979, **17,** 297). The antioxidant nature of BHT did not appear to be responsible for these effects, as they were not produced by single injection of butylated hydroxyanisole (BHA), vitamin E, *n*-propyl gallate (PG), ethoxyquin or *N,N'*-diphenyl-*p*-phenylenediamine, each at several dose levels (Omaye *et al. J. Toxicol. envir. Hlth* 1977, **3,** 829). Moreover, unlike BHT, equimolar doses of the antioxidants BHA, PG, pyrogallol, thiodipropionic acid, α-tocopherol and ascorbic acid failed to increase thymidine kinase activity in mouse lung (Witschi *et al. J. natn. Cancer Inst.* 1977, **58,** 301). Similarly, the increase in mouse lung weight produced by BHT could not be duplicated by six other antioxidants, four other substituted phenols, or the alcohol, acid or quinone metabolites of BHT (Malkinson, *Toxic. appl. Pharmac.* 1979, **49,** 551).

The changes induced by BHT in the mouse lung include a destruction of type I alveolar cells, followed by a proliferation of type II alveolar cells. Proliferation of type II cells is also a stage in the development of lung tumours after urethane injection, and it was not therefore entirely unexpected when BHT was shown to promote urethane carcinogenesis under some circumstances. Swiss–Webster or A/J mice given a single dose of 1 g urethane/kg, followed after 7 days by weekly ip injections of 250 mg BHT/kg, had after 11 or 13 wk developed significantly more tumours per lung than those given corn oil after urethane (Witschi *et al. loc. cit.*). The enhancing effect in Swiss–Webster mice was still evident when the initial urethane dose was lowered to 100 mg/kg, followed by 13 weekly injections of 300 mg BHT/kg, when the interval between urethane and BHT was increased to 6 wk, or when only four or eight BHT injections were given (Witschi & Lock, *Toxic. appl. Pharmac.* 1979, **50,** 391). BHT given by stomach tube in doses exceeding 100 mg/kg was also effective in producing lung-cell proliferation in the Swiss–Webster mouse, and 13 weekly oral doses of 300 mg/kg had a promoting effect on the lung tumours induced by a single ip dose of 1 g urethane/kg (*idem, Toxicology* 1978, **9,** 137).

Although BHT enhanced urethane carcinogenesis when given repeatedly after the urethane, a single injection of 300 mg BHT/kg followed after 1 hr–7 days by a single injection of urethane (1 g/kg) was without effect in Swiss–Webster mice (Witschi *et al. loc. cit.*). Thirteen weekly injections of 300 mg BHT/kg similarly failed to promote tumour formation in this strain when given before a single injection of 1 g urethane/kg (Witschi & Lock, *Toxic. appl. Pharmac.* 1979, **50,** 391). In contrast, in CFLP mice BHT (400 or 800 mg/kg) significantly enhanced the tumour yield when injected 6 or 7 days before 1 g urethane/kg (Bojan *et al. Bull. envir. Contam. Toxicol.* 1978, **20,** 573).

Three other mouse strains (C57BL, C3H and BALB/c), which have a low spontaneous incidence of lung adenomas, did not respond to the lung-tumour-promoting effects of repeated BHT injections starting a week after a single urethane injection. Moreover, in BALB/c mice given a single ip dose of BHT (400 mg/kg) only shortly before urethane, there was actually a reduction in the number of tumours formed. The urethane in this case was given as five ip injections each of 500 mg/kg over 38 hr, or at 0·3 or 0·5% in the drinking-water for 5 days, starting 12 or 18 hr respectively after the BHT (Witschi & Lock, *Toxic. appl. Pharmac.* 1979, **50,** 391). Weekly injections of BHT into Swiss–Webster mice pretreated with 0·9% sodium chloride also reduced the number of spontaneous pulmonary adenomas (Witschi *et al. loc. cit.*).

That BHT may enhance the pulmonary tumorigenicity of other substances besides urethane was suggested by a study in which 30 male BALB/c mice were given *N*-nitrosodiethylamine (NDEA) in their drinking-water for 7 wk and fed 0·75% BHT in their diet concurrently with the NDEA and subsequently until they were killed at 12 or 18 months of age (Clapp *et al. Fd Cosmet. Toxicol.* 1974, **12,** 367). However, when 400 mice were given the same treatment, after 12 months the enhancing effect of BHT was evident only in males, and in females a reduction occurred. After 18 months BHT had no influence on the incidence of lung tumours in either sex. An apparent BHT-promoted increase in squamous cell carcinomas of the forestomach in the preliminary study was not confirmed in the larger study in males, and in females BHT again had a protective effect (Clapp *et al. Proc. Am. Ass. Cancer Res.* 1975, **16,** 183; *idem, ibid* 1976, **17,** 168).

The preliminary study cited above also suggested that BHT alone may enhance spontaneous lung-tumour development. However, in the larger study BHT alone reduced the lung-adenoma incidence in males at 12 months, and had no effect on either sex at 18 months (Clapp *et al. Proc. Am. Ass. Cancer Res.* 1975, **16,** 182; *idem, ibid* 1976, **17,** 168). An apparent dose-related increase in lung tumours was reported in a Shell study in which $CF_1$ mice were fed 1000–5000 ppm BHT for 2 yr, although it was later suggested that this may have been due to aflatoxin

contamination of the diet (*Food Chemical News* 1978, 20 February, p. 25). An NCI bioassay involving dietary levels of 3000 and 6000 ppm BHT fed to rats and B6C3F1 mice for 105 wk was officially interpreted as giving negative results, but there was a non-dose-related increase in lung tumours in the female mice, and six tumours of the eye/lacrymal gland also occurred (*Food Chemical News* 1979, **21** (37), 40).

Witschi *et al.* (*Toxicology* 1981, **21**, 37) have now demonstrated that lung-tumour promotion by BHT is unlikely to be related to its ability to produce extensive lung proliferation, and that other antioxidants have little or no promoting effect. In addition they have confirmed that BHT may enhance lung-tumour development initiated by other carcinogens besides urethane, and that BHT administered before rather than after the urethane may also increase the lung-tumour incidence under certain conditions. To test the first hypothesis they used methylcyclopentadienyl manganese tricarbonyl (MMT) at a dose level (80 mg/kg) equivalent, in its ability to produce lung-cell proliferation, to 100–200 mg BHT/kg. Six weekly injections of MMT at this level failed to increase the numbers of tumours produced by 500 mg urethane/kg in A/J female mice. [No attempt was apparently made to use a higher MMT dose level, equivalent to 300 mg BHT/kg.] The findings were said to confirm those of a study in press, in which 100% oxygen, although producing a degree of cell proliferation in mouse lung similar to that produced by BHT, did not increase tumour multiplicity after urethane or increase the incidence of spontaneously occurring tumours.

The tumour-enhancing effect of BHT in the A/J mouse strain used was confirmed by giving a single ip injection of 500 mg urethane/kg, followed by eight weekly injections of 300 mg BHT/kg. Four months after the start of treatment there was an average lung-tumour incidence of 11·1/mouse, significantly more than the 7·2/mouse in those given urethane followed by corn oil. Of the other antioxidants investigated, BHA at 300 or 500 mg/kg (each for eight weekly injections) produced a slight but statistically insignificant increase in urethane-initiated tumours (8·4 and 8·9/mouse respectively) after 4 months. However, no promoting effect was evident from the lower BHA dose of 150 mg/kg, or from 1000 mg vitamin E/kg.

The other carcinogens tested as initiating agents (with BHT as 'promoter') were 3-methylcholanthrene (two sc injections, 1 wk apart each of 10 mg/kg) and *N*-nitrosodimethylamine (one sc injection of 7 mg/kg). Mice pretreated with these agents before being given four weekly injections of BHT (300 mg/kg) had after a further 3 months developed 3·5 and 2·5 times more lung tumours/mouse respectively than controls given corn oil instead of BHT.

The effects of urethane administration after BHT were first tested in mice given a single ip injection of 400 mg BHT/kg, and immediately afterwards given 150 or 1500 mg urethane/kg, either by single ip injection or by continuous infusion from an osmotic minipump during the period of lung-cell proliferation. No effect on lung-tumour incidence was apparent from either treatment. However, when urethane (500 or 1000 mg/kg) was injected 2 wk after BHT pretreatment (at which stage a mild interstitial fibrosis was still evident) there was a significant increase in lung tumours 4 months later.

Witschi *et al.* (*ibid* 1981, **21**, 95) have also demonstrated that BHT ingested in the diet may have the same effect as when injected or gavaged, whereas other antioxidants are again ineffective by this route. In addition they have provided further evidence that dietary BHT may enhance the spontaneous lung-tumour incidence. Male A/J mice were given a single ip injection of 500 mg urethane/kg and subsequently fed 0·75% dietary BHT, BHA or ethoxyquin for 24 hr, once weekly for 8 wk. After two further months the BHT-treated mice had developed an average of 10·4 tumours/lung, as compared with 7·1 in those given only urethane, 7·1 in the BHA group and 6·1 in the ethoxyquin group. Positive controls given eight weekly injections of 300 mg BHT/kg after urethane developed an average of 10·6 lung tumours/mouse, whereas only 6·6 tumours/mouse were found in those given urethane followed by repeated ip injection of corn oil. As the mice fed BHT or BHA had consumed only about half as much feed as controls during the dietary treatment periods, in a second experiment control groups were restricted for eight 24-hr periods to the same intake of untreated feed. Again 0·75% dietary BHT significantly increased the number of lung tumours, whether either 250 or 500 mg urethane/kg was given initially, whereas BHA was without effect. None of the antioxidants significantly increased the lung-tumour yield when fed in the same manner after a single ip dose of sodium chloride.

When mice given 250 or 500 mg urethane/kg were subsequently fed continuously for 8 wk with 0·75% BHT or BHA, after a further 8 wk BHT-treated mice showed a significant increase in lung-tumour yield, whereas BHA was without effect. BHT fed continuously for 8 wk after a single injection of 10 mg sodium chloride/kg also significantly increased the number of mice with spontaneous lung tumours, from 17 to 35%, and the number of tumours/mouse, from 0·1 to 0·4, BHA produced less marked and non-significant increases in these parameters. Prefeeding for 2 wk with 0·75% BHT before injection of 1 g urethane/kg did not markedly affect the tumour incidence, whereas 0·75% BHA pretreatment significantly reduced the number of tumours/mouse (from 20·3 to 7·7). Prefeeding with either antioxidant before sodium chloride injection had no significant effect. About 75% of the tumours in urethane-treated mice were alveolar cell tumours and the remainder were Clara cell tumours, and BHT did not produce a significant change in this ratio. The amount of BHT consumed was equivalent to about 625 mg/kg/treatment day when 0·75% was fed once weekly, and about 714 mg/kg/day when it was fed continuously, i.e. somewhat more than twice the 300 mg/kg used for ip injection. However, a study in press is said to have shown that six weekly ip injections of only 50 mg/kg are also effective in promoting urethane-induced tumours.

The relevance of these findings to man is so far uncertain. The BHT dose levels fed to mice in the last study were far higher than those normally ingested by humans from its use as a food additive, calculated to be 0·1–0·8 mg/kg/day on average and 2·9 mg/kg/day at maximum in the USA (*Food Chemical News* 1976, 8 November, p. 48). It is possible that BHT's enhanc-

ing effect on lung carcinogenesis in mice is a species-specific phenomenon, since it has not been reported in other species. In rats the ip injection of 500 mg BHA/kg produced a twofold increase in DNA synthesis in the lung in females, and no increase in males, in contrast to the 20–40-fold increase produced by the same dose in mice of both sexes (Larsen & Tarding, *Archs Toxicol.* 1978, Suppl. 1, 147). The lung tissue from rats given [$^{14}$C]BHT (400 mg/kg, ip) also contained much lower amounts of total and bound radioactivity than that from mice given the same dose, and the rats failed to develop lung lesions (Kehrer & Witschi, *Toxic. appl. Pharmac.* 1980, **53,** 333). Moreover, there was no evidence of treatment-related pathological changes or tumour induction in the lungs of rats fed 0·25 or 1·0% BHT for 104 wk (Hirose *et al. Fd Cosmet. Toxicol.* 1981, **19,** 147).

Studies with drug-metabolism inhibitors have suggested that a reactive metabolite of BHT, rather than the parent compound, is responsible for the lung damage in mice. Both SKF 525-A and piperonyl butoxide produced a dose-dependent inhibition of the increase in thymidine incorporation into pulmonary DNA caused by BHT in mice, and complete protection from 400 mg BHT/kg (ip) was provided by 10 and 400 mg/kg respectively of these agents. Lungs examined 5 days after treatment with BHT and 25 mg SKF 525-A/kg did not show any of the normal alveolar changes. These inhibitors gave complete protection when administered in the first hour (piperonyl butoxide) or two (SKF 525-A), and partial protection when administered up to 6–12 hr after the BHT. The BHT-induced increase in pulmonary DNA synthesis was also diminished by pretreatment with cobaltous chloride (60 mg/kg injected sc) and by BHT itself when a low dose (100 mg/kg) was given 20 or 48 hr, but not 6 hr, prior to a high dose (400 mg/kg). Administration of [$^{14}$C]BHT resulted in the covalent binding of radioactivity to lung tissue, and to a much lesser extent to liver and kidney tissue. Binding to lung tissue was a linear function of the BHT dose (in the range 50–600 mg/kg), and was significantly decreased by SKF 525-A (Kehrer & Witschi, *loc. cit.*).

Cedar terpenes (either as cedarwood shavings used as bedding or as injected sesquiterpenoids derived from cedarwood) also prevented BHT-induced lung damage in adult mice, and mice under 3 wk of age were resistant to this damage (Malkinson, *loc. cit.*). As neonatal mice possess low levels of drug-metabolizing enzymes, this finding too was consistent with the hypothesis that a BHT metabolite is responsible. However, the identity of this substance has not been established. Whether it is one of the metabolites also produced by man (*Cited in F.C.T.* 1979, **17,** 551) is therefore unknown.

It must also be remembered that dietary BHT protects against the action of some carcinogens, including polycyclic hydrocarbons and aromatic amines such as 2-acetylaminofluorene, when given at the same time as the carcinogen (*ibid* 1973 **11,** 328; Ulland *et al. Fd Cosmet. Toxicol.* 1973, **11,** 199; Wattenberg *et al. Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 1327). This protective action may be related to its ability to alter the microsomal metabolism of the carcinogens, and/or reduce their binding to DNA (Wattenberg *et al. loc. cit.*). On the other hand, there have been some indications that BHT may promote tumour development at other sites besides the lung. Rats fed 0·5% dietary BHT for up to 407 days after initial treatment with 2-acetylaminofluorene showed an increase in liver-tumour incidence, albeit far less pronounced than that produced by dietary phenobarbitone. BHT (6600 ppm in the diet) also slightly increased the number of intestinal tumours/rat after an initial dose of azoxymethane, although when the BHT was fed to animals concurrently receiving a weekly sc injection of azoxymethane it inhibited tumour development (Peraino *et al. Fd Cosmet. Toxicol.* 1977, **15,** 93; Weisburger *et al. ibid* 1977, **15,** 139). The mechanism for this apparent stimulation of carcinogenic action has still to be elucidated, although in the case of 2-acetylaminofluorene it may be related to the marked decrease in liver NADPH–cytochrome *P*-450 reductase activity which this compound and BHT both produce in the rat (Rikans *et al. ibid* 1981, **19,** 89).

Even if BHT's promotion of lung tumours in mice and liver and intestinal tumours in rats is in the future demonstrated to be relevant to man, it may still be well outweighed by BHT's protective effects. These include not only the inhibition of the action of certain chemical carcinogens, but also a prolongation of lifespan in mice fed 0·25% or 0·50% BHT in a semi-synthetic diet (Harman, *J. Geriat. Soc.* 1969, **17,** 721) and in the male offspring of female mice fed 0·1% BHT before and during gestation and weaning (Harman & Eddy, *Age* 1979, **2,** 109). This effect on lifespan, and BHT's ability to prevent the development of sarcomas in sarcoma-prone dogs (Franklyn, *Lancet* 1976, **i,** 1296) were attributed to its action as a free-radical inhibitor. BHT (0·7% in the diet) also completely eliminated the increased tumour incidence caused by 20% dietary saturated fat in rats, although it was far less effective in this respect when polyunsaturated fat was used (King *et al. J. natn. Cancer Inst.* 1979, **63,** 657). In addition pretreatment with dietary BHT protected against the cytotoxicity and reduced the covalent binding of aflatoxin $B_1$ in cultured rat hepatocytes (Salocks *et al. Toxic. appl. Pharmac.* 1981, **59,** 331). On balance, therefore, the conclusion might still be reached that BHT does more good than harm.

[C. Rostron—BIBRA]

## NITROSOPROLINE FATE AND FORMATION

*N*-Nitrosoproline (NPRO) was not carcinogenic in three studies reviewed by the IARC in 1978, although all were considered inadequate with regard to dose and/or duration (*Cited in F.C.T.* 1979, **17**, 167). A subsequent long-term study in which the high dose level of 2·5 g/litre was given in the drinking-water of 37 rats for 78 wk, followed by observation until death, also produced negative results (Mirvish *et al. J. natn. Cancer Inst.* 1980, **64**, 1435). In addition NPRO was non-mutagenic in an Ames test with *Salmonella typhimurium* TA1535 (*Cited in F.C.T.* 1978, **16**, 191) and in an X-linked recessive-lethal assay in *Drosophila melanogaster* (Nix *et al. Mutation Res.* 1980, **73**, 93). However, it was decarboxylated by the gut flora of two out of eight rats and one out of 15 rabbits to form *N*-nitrosopyrrolidine (NPYR), which is a hepatocarcinogen in rats (Kawabata & Miyakoshi, *IARC Scient. Publ.* no. 14, 1976, p. 261). In addition NPRO could transnitrosate other amines *in vitro* (Singer *et al. ibid* no. 19, 1978, p. 175). The latter reactions are of potential concern, as NPRO has been shown to be formed from nitrite and proline in the gastro-intestinal tract of rats (*Cited in F.C.T.* 1978, **16**, 71) and in foods such as cured meats. On the other hand, decarboxylation of NPRO to NPYR appears to occur to only a minor extent during the frying of bacon (*ibid* 1978, **16**, 389; Janzowski *et al. Fd Cosmet. Toxicol.* 1978, **16**, 343; *Cited in F.C.T.* 1980, **18**, 317).

A study in rats (Dailey *et al. Toxicology* 1978, **3**, 23) revealed that $^{14}$C-labelled NPRO was rapidly absorbed and excreted by fasted rats, about 98% being recovered as unchanged NPRO in the urine within 24 hr, and 1–2% appearing in the faeces after 24–48 hr. Fed rats had excreted 71–76% in the urine and 17–20% in the faeces 4–7 days after dosing. About 2% of the dose was exhaled by fed rats as $^{14}CO_2$ within 48 hr, and a further 3% was found in the carcass and tissues. In the first 4 hr, the bladder and kidneys of both fasted and fed rats contained much higher $^{14}$C levels than other organs and tissues examined.

Chu & Magee (*Cancer Res.* 1981, **41**, 3653) conducted a more detailed investigation, including a study of possible alkylation of cellular macromolecules. When rats fasted overnight were given 1 or 10 mg (*carboxy*-$^{14}$C)NPRO/kg orally, after 24 hr they had exhaled mean levels of only 0·66 and 0·45% of the dose, respectively, as $^{14}CO_2$, and 100·6 and 92·9%, respectively, of the radioactivity appeared in the urine as unchanged NPRO. When the same doses of (U-$^{14}$C)NPRO were given, 3·07 and 0·9%, respectively, appeared as $^{14}CO_2$ and 95·7 and 98·3% in the urine, again as the unchanged compound. The similar urinary excretion suggested that the difference in $^{14}CO_2$ production was not significant, and the lower or similar production of $^{14}CO_2$ from the [*carboxy*-$^{14}$C]NPRO suggested that very little or none was decarboxylated *in vivo*. No radioactive proline or its metabolites could be detected in the urine, indicating that no detectable denitrosation had occurred. However, since 96·4% of a dose of 1 mg [U-$^{14}$C]proline/kg was eliminated as $^{14}CO_2$, any proline produced by denitrosation *in vivo* would probably have been exhaled as $CO_2$ rather than excreted in the urine.

Rats that had not been starved overnight were given [U-$^{14}$C]NPRO (10 mg/kg) ip. The disappearance of radioactivity from the plasma was linear during the first 30 min after dosing, and only 13 and 2% of the maximum level at 5 min were left at 60 and 120 min respectively. Studies of the distribution of NPRO indicated that NPRO does penetrate the intracellular space to a limited extent. When starved rats were given a dose of about 1·8 mg [U-$^{14}$C] NPRO/kg orally and were killed after 4 or 8 hr, a total of only 0·82 and 0·33%, respectively, of the administered radioactivity was found in the liver. There was no increase in the $^{14}$C levels detected in the DNA from the liver sediment and the RNA from the liver supernatant from two treated rats compared with those from a control rat given non-radioactive NPRO. This indicated a lack of significant binding to nucleic acids. A very low level of radioactive binding to protein from liver supernatant was found, but quantities were insufficient to determine its identity. These findings contrasted with a recent report that NPRO is readily converted to an alkylating species by a nonenzymatic chemical-activating system consisting of ascorbic acid, ferrous ions, EDTA and molecular oxygen (Archer & Eng, *Chemico-Biol. Interactions* 1981, **33**, 207), a discrepancy probably explained by the very rapid elimination of NPRO from the body.

The endogenous formation of NPRO in a human male volunteer has been reported by Ohshima & Bartsch (*Cancer Res.* 1981, **41**, 3658). NPRO was detected in the urine after derivatization by combined gas–liquid chromatography–thermal energy analysis. After ingestion of 200 ml beetroot juice containing 260 mg nitrate, followed 30 min later by 500 mg proline, NPRO excretion in the urine reached a maximum of about 700 ng/hr after 6–8 hr and decreased over the following 12 hr. At 24 hr after ingestion of the precursors it was back to baseline levels. No increase above baseline levels occurred when either beetroot juice or proline was taken alone, or when 2 g ascorbic acid was ingested simultaneously with the proline. Ohshima & Bartsch (*loc. cit.*) went on to carry out more detailed studies using various doses of nitrate and proline. When beetroot juice containing 65, 130, 195, 260 or 325 mg nitrate was ingested with 500 mg proline, NPRO excretion increased exponentially with little or no increase at doses of up to 195 mg nitrate, but with five- and 15-fold increases at doses of 260 and 325 mg nitrate, respectively. Ingestion of different amounts of proline 30 min after 325 mg nitrate led to NPRO excretion in the range 2·7–30·0 μg, in direct proportion to the dose of proline. NPRO excretion in the urine within 24 hr of ingestion of 325 mg nitrate and 250 mg proline was in the range 14·0–15·9 μg (mean 14·9 μg), a rate some 5–7·5 times greater than when nitrate or proline alone was ingested. When 1 g ascorbic acid was ingested simultaneously with these levels of nitrate and proline urinary NPRO did not rise above baseline levels, whereas 500 mg α-tocopherol inhibited nitrosation *in vivo* by only about 50%. At the highest doses of nitrate (325 mg) and proline (500 mg) used, NPRO excretion was in the range 16·6–30·0 (mean

23·3) μg/24 hr, corresponding to 0·002 and 0·004% of the ingested nitrate and proline, respectively. Analysis of faeces of NPRO and NPYR, and of urine for NPYR or other volatile nitrosamines, provided no evidence that excretion of these compounds was increased. The NPRO presumably arose from nitrate re-secretion in the saliva, followed by its reduction to nitrite by the oral microflora and reaction with proline in the acidic medium of the stomach. It is suggested that the method of monitoring the excretion of *N*-nitroso compounds in the urine may be suitable for estimating the exposure to endogenously formed *N*-nitroso compounds of high-risk individuals, such as those with gastric achlorhydria.

The work of Chu & Magee (*loc. cit.*) suggests that ingested NPRO, or NPRO formed *in vivo*, is unlikely to present any great hazard, as NPYR formation and denitrosation of NPRO probably do not occur to any appreciable extent *in vivo*. The lack of nucleic-acid binding in the rat liver is particularly reassuring in view of the fact that NPYR is a hepatocarcinogen in rats. However, the study did use fasted rats, whereas far more NPRO was eliminated in the faeces of fed rats (Dailey *et al. loc. cit.*). In a fed animal there is thus presumably far more chance that decarboxylation to NPYR by the gut flora will occur. Moreover, the rat faeces were not examined to ascertain the absence of detectable NPYR, although such analysis was carried out in the study of a human subject by Ohshima & Bartsch (*loc. cit.*).

[C. Rostron—BIBRA]

## VITAMIN A—ON THE POSITIVE SIDE?

The possibility that vitamin A is one of those rare (or seldom recognized?) chemicals that have a positive contribution to offer in preventing cancer in man has excited a good deal of interest in recent years. The potential of retinoids in the treatment of cancer is being seriously pursued. However for most of the population (in well-nourished nations) an increase in vitamin A intake would certainly seem to be premature. Although all the lines of evidence do suggest that vitamin A is involved in the cancer process, they also indicate the complexity of that involvement.

The term vitamin A covers both the preformed vitamin in the form of retinyl esters, retinol and retinal, and its precursors, β-carotene and the other carotenoids. Most vitamin A in the British diet is derived from liver, dairy products or vitamin pills as preformed retinol or its esters but the remainder is derived from carrots or green leafy vegetables in the form of β-carotene or other carotenoids (*British Medical Journal* 1980, **281,** 957). This latter source is usually the main source of the vitamin for most people in poor countries and for some in richer countries. In the intestine (and possibly to a lesser extent in the liver) some of the ingested β-carotene is oxidized to retinal but the efficiency of this conversion is low (Peto *et al. Nature, Lond.* 1981, **290,** 201). Some dietary carotenoids are absorbed unchanged from the intestine, circulate in the blood and are stored in adipose tissue. Blood and adipose-tissue levels of carotenoids are influenced by factors such as disease but are largely directly dependent on the amounts of carotenoids recently ingested. Free retinol absorbed from the intestine is rapidly removed from circulation and stored in the liver which synthesizes retinol-binding protein (RBP) to transport controlled amounts of retinol to the tissues. The RBP leaves the liver when it has picked up a retinol molecule but if there is a shortage of retinol the RBP accumulates in the liver. The amount of circulating free retinol is normally insignificant compared with that in the bound form. Thus circulating retinol is not directly dependent on dietary intake within a broad range of dietary levels. However, the use of oral contraceptives substantially increases blood retinol levels and presumably there are other factors that have a similar or reverse effect (Peto *et al. loc. cit.*). Most people in developed countries consume enough retinol or carotene to avoid vitamin A deficiency but this leaves room for a wide range of intakes and of serum retinol levels.

Vitamin A is known to be important in the maintenance, growth and differentiation of epithelial tissues. Animals deficient in vitamin A have been shown to have increased susceptibility to various chemical carcinogens (*British Medical Journal loc. cit.*). Retinoids have been shown to suppress papilloma and carcinoma development (tumour promotion) in the mouse two-stage skin carcinogenesis assay, to inhibit mammary and bladder carcinogenesis in mice and rats and to inhibit the growth of some transplantable tumour lines (Nettesheim, *Can. med. Ass. J.* 1980, **122,** 757). *In vitro*, retinoids have been found to suppress the hyperplastic and metaplastic response of mouse prostate cultures to chemical carcinogens, to inhibit partially the transformation of C3H 10T½ cells by physical or chemical carcinogens and to inhibit the growth of some neoplastic and non-neoplastic cell lines (Nettesheim, *loc. cit.*). However, Schroder & Black (*J. natn. Cancer Inst.* 1980, **65,** 671) have cited several experimental studies that do not confirm the antitumour properties of retinoids. Such studies have demonstrated cell-activating properties of retinoids or enhancement of tumorigenic responses to chemical or UV irradiation by high doses of topically applied retinoids. Further, Schroder & Black point out that retinoid-induced cell activation and enhanced expression of the transformed cell phenotype have also been noted in some *in vitro* studies.

Despite these indications that retinoids may have tumour-enhancing properties under certain conditions, the main body of experimental evidence still seems to support a protective role for the vitamin. The most important evidence of the protective role of vitamin A comes from epidemiological studies. There have been two distinct lines of investigation concen-

trating either on serum vitamin A levels of cancer patients compared with controls or on dietary intake of vitamin A/carotenoid sources.

*Serum vitamin A*

Basu *et al.* (*Br. J. Cancer* 1976, **33**, 119) found that 28 patients diagnosed as having bronchial carcinoma had significantly lower (mean, 45·6 μg/100 ml) plasma vitamin A levels than did the two groups of controls (means, 64·3 and 68·4 μg/100 ml, for nine patients with non-malignant lung disease and ten healthy subjects, respectively). Patients with squamous or oat-cell carcinoma had significantly lower plasma vitamin A levels than did those with large-cell undifferentiated carcinoma. The same group (Atukorala *et al. ibid* 1979, **40**, 927) carried out a further similar study involving 22 male and four female lung-cancer patients and 21 controls (15 males and six females with non-malignant diseases). Again serum levels of vitamin A in the cancer patients were significantly lower than in the controls but for serum β-carotene the difference was not significant. No significant differences in vitamin A levels were found when the cancer patients were grouped according to tumour type. The serum concentration of RBP was also significantly lower in cancer patients than in controls and was significantly correlated with serum vitamin A in the cancer patients but not in the controls. The mean serum concentration of zinc was lower and that of copper was higher in cancer patients than in controls. The serum zinc concentrations were positively correlated both with vitamin A and with RBP in the cancer patients but not in the controls. Atukorala *et al.* (*loc. cit.*) did not consider that these results necessarily provided support for the possibility of a role for vitamin A in lung cancer. They thought that the low levels of vitamin A in the plasma of the cancer patients might have resulted from the growth of the tumour, especially in view of the high incidence of lung-cancer in Britain where vitamin A deficiency hardly occurs. They point out that their results are consistent with suggestions that zinc may be involved in the mobilization of vitamin A and possibly in the synthesis of RBP. It also seems that rapidly growing tumour tissue may increase the body's requirement for zinc and could thus be responsible for the low levels of circulating vitamin A. Such arguments are of little value, however, if indeed the low serum levels of vitamin A are a predisposing factor in lung cancer.

The evidence emerging from more recent prospective studies is much less equivocal. Wald *et al.* (*Lancet* 1980, **II**, 813) investigated the relationship between serum vitamin A levels and subsequent cancer incidence. Between March 1975 and December 1978 they collected blood from 16,000 men aged 35–64 and stored the serum at −40°C. By the end of 1979, 86 cancer cases had been identified and 172 controls, matched for age, smoking habits and date of blood sampling, were selected. The mean serum retinol level for the cancer cases was significantly lower than that for the controls, the difference being greatest in those with lung cancers. The relative risk of developing cancer fell with increasing blood-retinol level and this trend was statistically significant. The mean retinol levels of men for whom there was a suspicion of cancer when the samples were taken were not lower than those of other cancer patients, suggesting that the low vitamin A levels are not just a metabolic consequence of the tumour. Wald *et al.* concluded that serum retinol values in man have predictive value for subsequent cancer.

Amongst the correspondence generated by this Wald *et al.* paper was a letter from McLaren (*ibid* 1980, **II**, 1144) who pointed out that the vitamin A levels found by Wald *et al.* (means 183–231 IU/100 ml) were not low by international standards. McLaren considered that since there was no indication that any of the individuals was deficient in vitamin A it was misleading to describe the cancer patients as having low serum vitamin A levels. Briggs (*ibid* 1980, **II**, 1145) was led to speculate that since women using oral contraceptives have high serum vitamin A levels this might afford them some protection from cancer.

Kark *et al.* (*J. natn. Cancer Inst.* 1981, **66**, 7) carried out a prospective study of serum vitamin A levels in Georgia, USA. Blood samples were taken from 3102 people and sera were stored at −18°C. During the following 14 yr, 85 cancer cases developed and 162 matched controls were selected. The cancer cases had significantly lower serum retinol levels (41·27 μg/100 ml) than did the controls (46·86 μg/100 ml) but the difference was greater for males than for females.

*Dietary studies*

During a 5-yr follow-up period of 8278 men who had previously reported their cigarette-smoking and dietary habits, Bjelke (*Int. J. Cancer* 1975, **15**, 561) found 36 cases of cancer of the bronchus and lung. Each of the men was assigned to a smoking category and was given a vitamin A index on the basis of the frequency with which he ate certain items. Several vegetables, particularly carrots, milk and to a smaller extent eggs were the chief contributors to the vitamin A index. A lower lung-cancer rate was found for those with high values of the vitamin A index (greater vitamin A intake) at all levels of cigarette smoking in all age groups. Only four lung-cancer cases were found in those who had never smoked so that it was not possible to draw any conclusions about the effects of vitamin A on lung-cancer risks in nonsmokers.

Hirayama (*Nutr. Cancer* 1979, **1**, 67) carried out a prospective study of 265,118 adults who were followed up for 10 yr after being interviewed about their smoking and dietary habits. He found a much lower risk of lung cancer in male and female smokers and nonsmokers who consumed green/yellow vegetables daily than in those who ate them less frequently. A decreased risk of cancer of the prostate and a slightly decreased risk of stomach cancer were also associated with daily consumption of green/yellow vegetables. The decreased risk was attributed to the protective role of vitamin A, but clearly such effects might be produced by some vegetable constituent other than vitamin A or by some other dietary factor correlated with vegetable consumption.

There have also been a number of retrospective dietary studies that involve asking cancer patients and matched controls about their past diet. However, the reliability of such studies is hampered by the possibility that the disease may cause memory of past diet

to be coloured. Mettlin *et al.* (*J. natn. Cancer Inst.* 1979, **62**, 1435) obtained dietary and smoking histories for 292 American white male patients with histologically confirmed cancer of the trachea, bronchi or lungs and for 801 matched controls. Milk and carrots were the main contributors to the calculated vitamin A index. The incidence of lung cancer decreased as the value of the vitamin A index increased. This pattern was also found in the age-adjusted relative risk for each smoking category. The effect of vitamin A was most evident among heavy smokers. Another retrospective study, by Gregor *et al.* (*Nutr. Cancer* 1980, **2**, 93), involved 82 male and 22 female patients with histologically proven lung cancer and 112 male and 64 female controls with non-malignant disease. Patients with chronic obstructive airways disease were excluded since this group had previously been shown to have low vitamin A levels. The estimated vitamin A intake was significantly lower in male cancer patients than in male controls but it was significantly higher in female cancer cases than in controls. The differences in intake arose chiefly from patterns of consumption of liver and vitamin A supplements. When the subjects were divided into three groups based on estimated total vitamin A intakes there was a statistically significant decrease in lung-cancer risk with increasing vitamin A intake in males. Among the females the trend was in the opposite direction but was not statistically significant.

Smith & Jick (*Cancer, N.Y.* 1978, **42**, 808) compared the frequency of regular use of vitamin A preparations among 800 newly diagnosed cancer patients with that among 3433 patients with non-malignant diseases. They found a suggestion of a negative association between vitamin A consumption and cancer risk among the males but not among the females nor when the two sexes were considered together. In the light of the data from Gregor *et al.* (*loc. cit.*) it seems possible that there might be a sex difference in the influence of vitamin A on cancer risk.

With the puzzling exception of the females in these studies there seems to be a consistently decreased risk of cancer among people with high intakes of vitamin A and/or $\beta$-carotene. Peto *et al.* (*loc. cit.*) cite a number of other studies that have shown this same trend in relation to cancers of various organs and vitamin A, $\beta$-carotene or green-vegetable intakes. It is possible that such results are simply a reflection of the intake of some other protective factor or the avoidance of some adverse factor. Although not yet firmly established, a link between serum vitamin A levels and cancer risk also seems likely. The gaps in our knowledge of the factors that affect serum vitamin A levels appear to present the next stumbling block in the way of making practical use of such a link.

[M. A. Gray—BIBRA]

## FURTHER COMMENTS ON OESOPHAGEAL CANCER

While making no conclusive contribution towards clarification of the aetiology of oesophageal cancer, a few recently published papers have touched on some of the questions raised in our recent review of data relating to areas characterized by a high incidence of this type of tumour (*Cited in F.C.T.* 1981, **19**, 781). Mutagenic studies on apple brandies consumed in Western France have been more fully reported, an attempt has been made to identify risk factors in an area of relatively high incidence in India and the possible involvement of fungal activity in oesophageal cancer has received further attention. In addition the interesting question of the possible role of zinc in tumour development has been brought to the fore in a laboratory study of oesophageal-tumour induction.

Loquet *et al.* have now reported (*Mutation Res.* 1981, **88**, 155) that their apple brandy study covered Ames tests not only on 30 home-made apple brandies from various parts of Normandy, 18 commercial apple brandies and 28 other commercial spirits but also on four known constituents of distilled beverages—acrolein, $\gamma$-butyrolactone, furfural and glycidol. None of the beverages were mutagenic to *Salmonella typhimurium* strains TA98 or TA100 when tested at a level of 200 $\mu$l/culture, with or without metabolic activation, but when alcoholic, aqueous and non-volatile fractions were tested, using the same volume of sample, the aqueous fractions of two commercial apple brandies were weakly mutagenic when tested in the absence of S-8 mix. Weak mutagenicity was also shown by the alcoholic fractions of five of the other spirits (but only after metabolic activation) and by 11 fractions derived from the home-made apple brandies. In the latter case, the 11 positive results were distributed across all three fractions and occurred in activated or non-activated cultures or sometimes in both. This wide range of results suggests the involvement of a variety of components in the apparent mutagenic potential of these beverages. Of the common constituents tested separately in TA98, TA100 or TA1535, only glycidol produced any positive results—in TA100 and TA1535 without metabolic activation. The authors indicate that glycidol may be present in apple brandies at levels of about 10 mg/litre, but the levels were not determined in the beverage samples included in this study.

We noted previously the wide variation in male to female oesophageal cancer ratios in different high-incidence areas. In Bombay the incidence of this tumour in no way compares with the 100–200 cases/100,000 recorded in the worst-affected areas of Iran and China; nevertheless in that city it is reported to be the third most commonly encountered cancer and the incidence shows little difference between the sexes. Jussawalla (*J. Cancer Res. clin. Oncol.* 1981, **99**, 29) has cited incidence rates of 15 and 11/100,000 for Bombay males and females respectively. This author compared the occurrence of 'pan-chewing' tobacco

smoking and alcohol consumption in 649 oesophageal-cancer patients with that in 649 controls matched for age, sex and religion, and he identified all three activities as possible high-risk factors for the disease in this area. The main constituents of the pan quid are betel leaf and nut, slaked lime, catechu and (sometimes) tobacco. Pan-chewing seems to offer a greater risk when the pan is tobacco-free, probably because the inclusion of tobacco makes the liquid extract too pungent to be swallowed. In contrast, smoking plus pan-chewing has an additive or even synergistic effect on the degree of risk, as also seems to be the case with a combination of smoking and alcohol. While pan-chewing is widely practised by both men and women, tobacco smoking and alcohol consumption are largely restricted to the male population in Bombay. The balance may be maintained, however, by the prevalence among Indian women of vitamin and iron deficiencies, which have been associated elsewhere with oesophageal cancer.

Suggestions about the possible causes of the incredibly high incidence of carcinoma of the oesophagus in Linhsien County in the Chinese province of Northern Honan have included a nitrosamine-rich component of the local diet (*Cited in F.C.T.* 1981, **19,** 782). A related line of enquiry has recently been reported jointly by workers from the Chinese Academy of Medical Sciences, Beijing, and The Memorial Sloan-Kettering Cancer Center, New York (Hsia *et al. Proc. natn. Acad. Sci. U.S.A.* 1981, **78,** 1878). Investigations in Northern Honan have demonstrated that fungal infections, particularly involving Candida species, are often associated with hyperplasia or dysplasia of the oesophageal epithelium in patients showing premalignant changes or early oesophageal carcinoma. Subsequent *in vitro* studies have established that *Candida albicans* can promote the formation of *N*-nitroso-*N*-methylbenzylamine (NMBA) from its precursors, *N*-methylbenzylamine (MBA) and sodium nitrite. NMBA is a specific inducer of oesophageal tumours in rodents, while nitrite and nitrate are commonly found in the drinking-water and foods in Linhsien County and MBA has also been identified in a variety of local foods, especially vegetables. Moreover, the thickening of the oesophageal epithelium that results from heavy Candida infection may lead to the accumulation of acidic products in the tissue, with consequent reduction of local pH to levels at which the nitrosation reaction readily occurs. It thus seems possible that a nitrosamine may be formed not merely in foods prior to their consumption but—more significantly—in close proximity to its target tissue.

Low dietary levels of certain vitamins have been mentioned in connection with the poor nutritional status of inhabitants of the areas of high oesophageal-cancer incidence along the Caspian littoral (*Cited in F.C.T.* 1981, **19,** 782). Another essential dietary component possibly involved in this problem is zinc. Reporting a laboratory study in rats, van Rensburg *et al.* (*Nutr. Rep. Int.* 1980, **22,** 891) have commented that the zinc level is likely to be low in diets based predominantly on wheat, as is the case in Iran. Not only is the zinc content lower in wheat than in some other important carbohydrate sources, but intestinal absorption of the element may be depressed by the intervention of other constituents of the grain, particularly phytate. Zinc deficiency has certainly been demonstrated among Iranian population groups (*Cited in F.C.T.* 1972, **10,** 580).

The work reported by van Rensburg *et al.* (*loc. cit.*) demonstrated that a condition of zinc deficiency increased the susceptibility of rats to the induction of oesophageal tumours by a series of six 2-mg/kg doses of NMBA given subcutaneously or intragastrically over a 3-wk period. The zinc deficiency, brought about by the feeding of a phytate-enriched diet, was reflected in a marked lowering of the plasma-zinc level but had no effect on growth rate. One hundred days after NMBA treatment there were more tumours in the phytate-fed rats than in similarly treated rats fed the control diet, and there was a negative correlation between plasma-zinc levels and the average diameters of the tumours. The difference in tumour incidence was eliminated when rats from both groups were maintained for a further 100 days on the control diet, which was adequate in zinc, but the mean tumour diameter remained higher in the rats previously fed excess phytate. The lesions classed as 'tumours' in this study included all nodules exceeding 0·3 mm in diameter; some of the larger nodules were well-developed papillomas showing, in some cases, early signs of malignant transformation, but in this fairly short study none had progressed to carcinomas.

One possible interpretation of these findings is that a low zinc level in the body may hasten the appearance and development of incipient oesophageal tumours, and the authors conclude that zinc intakes that are just adequate to ensure optimal growth are insufficient to maintain the maximal resistance to oesophageal carcinogenesis. In this connection it is interesting to note a report of low levels of zinc in the serum and hair of Chinese men who were still well nourished but had early oesophageal carcinomas (Lin *et al. Nutr. Rep. Int.* 1977, **15,** 635). However, the relationship between zinc and tumour development is far from clear. Some of the evidence is conflicting, but studies on changes in the tissue distribution of zinc in man in the face of malignant disease raise interesting questions about the role played by this element. For example, comparison of zinc concentrations in various organs—particularly the demonstration that concentrations of zinc are higher in healthy liver tissue than in other parts of the same liver showing macro- or microcarcinomatous invasion—suggests that an increase in zinc content may be part of a normal tissue's biochemical defence against invasion by malignant cells (Griffith *et al. Nature, Lond.* 1973, **241,** 60; Wright & Dormandy, *ibid* 1972, **237,** 166).

In a later issue we hope to draw together the available data relating to the possible sequence of events underlying zinc's involvement in the development of malignancy and in certain other cellular interactions. In the specific context of oesophageal cancer, too, dietary zinc may well deserve further attention.

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# ABSTRACTS AND COMMENTS

## Success is sweet for aspartame

Ishii, H., Koshimizu, T., Usami, S. & Fujimoto, T. (1981). Toxicity of aspartame and its diketopiperazine for Wistar rats by dietary administration for 104 weeks. *Toxicology* **21**, 91.

Following extensive toxicological evaluation of the 'new' low-calorie sweetening agent, aspartame (L-aspartyl-L-phenylalanyl methyl ester), is now approved for marketing in nine countries and applications for approval have been submitted in many more (*Sweeteners Bulletin*, August 1981, no. 1, p. 6). Nevertheless, its progression from test tube to table top has not been entirely trouble-free. This is particularly true in the United States where a Public Board of Inquiry, established to investigate various aspects of the toxicological data submitted by the petitioner, expressed concern over the possible carcinogenicity of aspartame (Department of Health and Human Services, Food and Drug Administration [Docket No. 75F-0355]). However on reanalysing the same data, the Bureau of Foods rejected the Board's findings and recommendations (*Food Chemical News* 1981, **23** (41), 42 & **23** (42), 31) and has subsequently removed the 7-year stay on aspartame's food additive order (*Federal Register* 1981, **46**, 38285). A recent Japanese study on the long-term safety of this additive and its contaminating degradation product, diketopiperazine, has now been published in an abridged form.

Five groups of 86 rats of each sex were fed aspartame (APM), or aspartame plus diketopiperazine (DKP) in the ratio of 3:1, at dietary levels of up to approximately 10%, resulting in daily doses of 0, 1, 2 or 4 g APM/kg body weight or 4 g APM + DKP/kg. Ten and 16 rats of each sex from each group were killed after 26 and 52 wk, respectively, and all surviving animals were killed after 104 wk. No dose-related effects on mortality were demonstrated. Weekly records of body weights and food and water consumption revealed a dose-dependent depression of growth in treated males, with the exception of those in the lowest dose group, and all females after 20 wk of treatment, compared with controls. This corresponded to reduced food intakes and may have resulted from absorption of the amino acids freed by hydrolysis of aspartame in the gut. An increase in urinary specific gravity and a decrease in urinary pH was seen in the groups given APM + DKP after 26 and 52 wk. Urinary pH was also lowered at 104 wk in animals receiving APM + DKP, at 52 wk in females given 2 or 4 g APM/kg and at 26 wk in females given 4 g APM/kg. It was considered possible that these effects, and a slight increase in relative kidney weight in rats receiving 4 g APM + DKP/kg, could be attributed to the excretion of acidic DKP. There were no treatment-related haemocytological effects, but a tendency towards lowered total cholesterol levels in those consuming 4 g APM or APM + DKP/kg was recorded. There were also minor increases in relative spleen weights at 26 and/or 52 wk in males given 2 g APM/kg and in both sexes given 4 g APM or APM + DKP/kg. This appeared to be without adverse effect on the animals. The observed dose-related rise in urinary calcium levels of rats given 2 or 4 g APM/kg or APM + DKP was considered to be due to enhanced calcium absorption. (High-protein diets cause increases in calcium absorption and excretion in rats.) This was associated with a slight dose-dependent increase in focal mineralization of the renal pelvis at 104 wk and both findings were thought to be without biological significance.

Thus, the authors concluded that no toxic effects were demonstrated in this study.

## Food colourings—no effect on hyperactive children

Mattes, J. A. & Gittelman, R. (1981). Effects of artificial food colourings in children with hyperactive symptoms. A critical review and results of a controlled study. *Archs gen. Psychiat.* **38**, 714.

The paucity of data from controlled studies validating Dr Feingold's excessively publicized beliefs that food additives are responsible for hyperkinesis in children was highlighted by the US National Advisory Committee on Hyperkinesis and Food Additives in its 1975 preliminary report (*Food Chemical News* 1975, **16** (45), 39). In its later report (The Nutrition Foundation, Inc., Washington, 1980) the Committee concluded that studies of "reasonable research design" had not validated Dr Feingold's claims. Similarly, the EEC Commission's Working Group on Adverse Reactions to Ingested Additives (Report No. III/556/81-EN, Brussels, 1981) has emphasized the highly equivocal nature of the evidence claimed to support the theory. In the paper cited above the authors present a comprehensive and critical summary of the relevant literature and then report the lack of effects of artificial food colourings on children with hyperactive or other symptoms when tested in a controlled study.

Thirteen children aged 4–13 yr, who were reported by their parents to respond markedly to artificial food colourings and were currently following the Feingold diet, were given placebo cookies (containing no colourings) in an initial 1-wk, non-blind trial. Two children who reacted adversely were excluded from further study. The clinical characteristics of the eleven children remaining varied: five were diagnosed as currently hyperactive, three had histories (reported by parents) diagnosed retrospectively as hyperactivity and three had histories of some other behavioural difficulty.

These children were given for 1 wk either placebo or test cookies, which were shown to be indistinguishable from placebo cookies, but contained 13 mg of a

mixture of all FDA-approved food colourings in proportions representing normal consumption patterns. The cookies were given at breakfast, lunch and after school. Starting with one cookie on the first day, an additional cookie was given each day to a maximum of six on the sixth and seventh days to all but three children (who failed to consume more than four daily). The trial was carried out 'double blind' with neither the children nor anyone involved in their care or evaluation being aware of the type of cookie given. The subjects remained untreated during the following week and then the test procedure was repeated giving the alternative treatment during the final week of the experiment.

Before commencing the investigation and during both treatment periods ratings of the child's behaviour were obtained from parents, teachers and psychiatrists using various standard scoring systems, a distractability test and a scale specially developed to assess typical hyperactive symptoms.

No treatment effects were demonstrated and the order of treatment did not affect the result. Similarly negative results were obtained when the data were analysed excluding those for children who consumed only four cookies per day or those not diagnosed as hyperactive. Thus this controlled study in a small selected group failed to yield positive support for Feingold's theory and clearly showed that food-colouring supplementation of the Feingold diet had no effects on the behaviour of these children.

**Possible progress in evaluating sulphite safety**

Gunnison, A. F., Farruggella, T. J., Chiang, G., Dulak, L., Zaccardi, J. & Birkner, J. (1981). A sulphite-oxidase-deficient rat model: metabolic characterization. *Fd Cosmet. Toxicol.* **19,** 209.

Gunnison, A. F., Dulak, L., Chiang, G., Zaccardi, J. & Farruggella, T. J. (1981). A sulphite-oxidase-deficient model: subchronic toxicology. *Fd Cosmet. Toxicol.* **19,** 221.

Those preservatives that depend for their function on the generation of sulphur dioxide produce problems for the regulatory agencies, since their technological necessity is associated with an equivocal toxicological profile. It appears that some wine drinkers may be regularly exposed to sulphur dioxide at levels above the current ADI of 0·7 mg/kg body weight set by JECFA in 1973 (*Food Chemical News* 1975, 27 October, p. 42; Report of the 12th Session of the Codex Alimentarius Committee on Food Additives; Alinorm 79/12, FAO/WHO, Rome, 1979). Concern over the health consequences of exposures of this order may be warranted since the ADI is based on the results of feeding studies in the rat conducted by Til *et al.* (*Fd Cosmet. Toxicol.* 1972, **10,** 291). In mammals, the principal metabolic defence against systemic sulphite is its conversion to the relatively non-toxic sulphate ion by a molybdenum-containing mitochondrial enzyme, sulphite oxidase. This enzyme is present at its highest level in the liver. Of the known species differences in sulphite-oxidase activity, the most worrying is that man possesses only about 5–10% of the hepatic sulphite-oxidase activity of the rat (Johnson & Rajagopalan, *J. clin. Invest.* 1976, **58,** 551 & 543). Gunnison and his colleagues at the New York University Medical Center, who have been studying the sulphite-oxidase system in laboratory animals for a number of years, are now working with an interesting rat model which may prove of great value in more accurately defining man's susceptibility to ingested sulphite. They are building upon results of earlier studies which demonstrated that rat tissues can be depleted of sulphite oxidase by the administration of a high tungsten–low molybdenum diet (Johnson *et al. J. biol. Chem.* 1974, **249,** 859).

In the present experiments the sulphite-oxidase level of the livers of female Wistar rats were shown to be rapidly reduced when a special low-molybdenum (0·058 ppm) diet was given in conjunction with 200 ppm tungsten in the drinking-water. Steady-state enzyme levels, which were reached after some 4 wk of treatment, were dependent on the relative levels of the two metals that were given subsequently. A tungsten:molybdenum ratio of 11,600:1 reduced levels to below the limit of detection, whereas a ratio of 3100:1 (corresponding to 100–120 ppm tungsten in the drinking-water) produced a steady-state oxidase activity that was 1% of those of normal adult animals. The animals could maintain normal sulphite-oxidase levels if they were given a low-molybdenum diet and unadulterated drinking-water. The biochemical effect of tungsten could be completely eliminated by the addition of extra molybdenum (4 ppm) to the low-molybdenum diet, the tungsten:molybdenum ratio in this instance being 50:1 for the first 28 days of treatment and 25:1 thereafter.

In rats exposed only to endogenous sulphite generated from the breakdown of sulphur-containing amino acids, there was an inverse relationship between hepatic sulphite-oxidase activity and levels of both *S*-sulphonate in tissues and of *S*-sulphonate and thiosulphate in urine. With respect to exogenous sulphite given by gavage, at relatively low systemic doses (up to 10,000 nmol/ml × min) the doses required to produce any specific systemic level of sulphite (as measured by plasma sulphonate) were some twenty times higher in the normal rat than in the animals with 1% sulphite-oxidase activity as determined by *in vitro* assay. These results were consistent with the view that the rate-limiting step for sulphite oxidation is the rate of diffusion into the mitochondria and not the amount of sulphite oxidase in the mitochondria.

In an initial programme of toxicity studies, three groups of rats, in which low (1%) hepatic sulphite-oxidase levels were induced and maintained as above, were given 0, 25 or 50 mM-metabisulphite in their drinking-water for 9 wk and were compared with control groups of normal sulphite-oxidase status. Four control groups were used in total; one was given an ordinary laboratory diet, and three were maintained on the special low molybdenum diet used for the treatment (sulphite-oxidase-deficient) groups. Two of these last three control groups were in addition given drinking-water containing tungsten, at levels similar to those given to sulphite-oxidase-deficient rats, and 4 ppm molybdenum together with either 12·5 or 25 mM-$Na_2SO_4$, which provided concentrations of

sodium equimolar to the 25 and 50 mM-sulphite-treated, sulphite-oxidase-deficient groups. The $Na_2SO_4$-treated control groups were matched for weight and water consumption with their sulphite-treated counterparts and were pair watered. For further comparison four groups of rats with normal sulphite-oxidase activity were given the low-molybdenum diet incorporated with either 0, 1, 2 or 6% sodium metabisulphite. Diets containing metabisulphite, but not the control diet, were supplemented with 50 ppm thiamine.

On the basis of tissue sulphonate levels, the authors calculated that the endogenous sulphite produced by sulphite-oxidase-deficient rats given no sulphite in the diet resulted in systemic sulphite exposures comparable to those in normal rats given a diet containing 2% metabisulphite. On the same basis. 50 mM-sulphite (about 3·5 mmol/kg/day) in the drinking-water of the sulphite-oxidase-deficient rats was equivalent to 6% dietary metabisulphite (which provided about 25 mmol $SO_3^{--}$/kg/day). The sulphite-oxidase-deficient rats were however by no means clinically identical to normal animals with equivalent systemic sulphite levels. The latter suffered from severe anaemia, a finding also reported by Til *et al.* (*loc. cit.*), whereas the sulphite-oxidase-deficient rats had normal haematological profiles. The levels of thiamine in the livers of sulphite-oxidase-deficient animals were within normal limits, but in the normal animals given 6% dietary metabisulphite there was a slight but statistically not significant thiamine deficiency despite the thiamine supplementation of the diet. It seems therefore that neither anaemia nor thiamine deficiency are a consequence of elevated systemic sulphite levels.

The only other experimental finding of note in these initial studies was a low incidence of mammary adenocarcinoma in the groups of sulphite-oxidase-deficient animals. Although the incidence of this tumour type, 4/149 in the oxidase-deficient animals compared with 0/143 in the controls, was not statistically significant, and there was no dose-relationship, the fact that these occurred in rats that had been treated for only 40–65 days and, being aged less than 5 months, were unlikely to have developed tumours spontaneously, lead the investigators to suggest tentatively that the tumours were treatment related.

The results of the somewhat cursory metabolic study of the sulphite-oxidase-deficient animals correlate fairly well with what is known about sulphite-oxidase-deficiency in humans. However, the small number of affected individuals that have been studied did have larger amounts of cysteine *S*-sulphonate and inorganic thiosulphates in their urine than would have been expected on the basis of the amount of *S*-sulphonate metabolites excreted by the sulphite-oxidase-deficient rats in this study. This suggests that hepatic levels of sulphite oxidase may be lower in affected humans than in the rat model described here. In normal humans, sulphite and sulphonated cysteine are not detectable in the urine.

The animal model described in these two papers is certainly interesting and should have great potential in hazard evaluation. However it is a complex experimental system and there is still some way to go before the results from it can be viewed with confidence. Further studies to investigate the reproducibility of tumorigenicity in the model system are obviously awaited with great interest. In the light of sulphite's well-established mutagenic activity in lower organisms (*Food Chemical News* 1977, 2 May, p. 20), studies of mutagenicity in oxidase-deficient animals would appear to deserve a high priority in any future work programmes.

### Ethanol—a dominant lethal test

Chauhan, P. S., Aravindakshan, M., Kumar, N. S. & Sundaram, K. (1980). Failure of ethanol to induce dominant lethal mutations in Wistar male rats. *Mutation Res.* **79,** 263.

Chronic alcoholics show a significant increase in chromosomal aberrations in their peripheral blood (De Torok, *Ann. N.Y. Acad. Sci.* 1972, **197,** 90; *Cited in F.C.T.* 1982, **20,** 237). Ethanol itself has been reported to induce dominant lethal mutations when fed to male mice (*ibid* 1975, **13,** 581). In contrast, we now report a study where feeding ethanol to male rats did not induce dominant lethal mutations.

Inbred albino male Wistar rats, 6–7 wk old, were randomly divided into five groups and caged individually. One group was given 15% ethanol in drinking-water (*ad lib.*) for 5 days after which the concentration was increased to 20% for 30 days. A second group received 15% for 5 days, 20% for five days, 25% for 10 days and 30% for a further 15 days. A third group received 30% for 4 days only, a fourth group were untreated and a fifth group was exposed to 200 R X-rays (100 R/min) 1 hr before mating. Soon after each treatment had ended, each male was paired with two or three female rats (10–12 wk old) which were replaced after a week, and eight sequential weekly pairings were undertaken. Ten to 11 days after their removal from the males, the females were killed and examined for live and dead implantations. There were no significant differences in pregnancy rates between the control group and the 30%-ethanol groups. Females mated with males from the group receiving 15–20% ethanol showed a relatively low pregnancy rate, which was mainly due to two males who were only successful in impregnating the females on 7/24 and 2/24 occasions, respectively. Examination of these two rats failed to reveal evidence of gross pathology of any organ and their testes weights were normal. No cytology of the testes was undertaken. The females mated with irradiated males showed a high incidence of dead implantations and a reduction in the number of live implantations. There were no significant differences between the controls and the females mated with ethanol-treated males in the numbers of corpora lutea, total live and dead implantations or total live implantations except during the fifth week. The authors considered that significant decreases in the numbers of implantations during the fifth week of mating could not be associated with ethanol consumption since there were no effects at any other matings.

### Nitrosamines in edible oils

Sen, N. P. & Seaman, S. (1981). An investigation into the possible presence of volatile *N*-nitrosamines in

cooking oils, margarine, and butter. *J. agric. Fd Chem.* **29,** 787.

Volatile *N*-nitrosamines have been found in a number of foods and beverages (Goff & Fine, *Fd Cosmet. Toxicol.* 1979, **17,** 569; Scanlan *et al. ibid* 1980, **18,** 27; Libbey *et al. ibid* 1980, **18,** 459; *IARC Scient. Publ.* no. 14, 1976). It has been suggested that their formation is due to the nitrosation of amines by nitrates and nitrites contained in the foods (either naturally or as additives) or, in foods such as dried milks that are prepared by direct drying, by the reaction between secondary and/or tertiary amines in the food and the oxides of nitrogen that are formed during fuel combustion (Libbey *et al. loc. cit.*). The presence of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) at levels of up to 27·8 ppb in various vegetable oils (particularly olive, soya-bean and plant-germ oils) has been reported by workers in Germany (Hedler *et al. J. Am. Oil Chem. Soc.* 1979, **56,** 68). The results of a study carried out to determine the levels of volatile *N*-nitrosamines present in edible fats and oils sold in Canada have now been published.

Six samples of lard, ten of butter, 14 of margarine and 38 of various vegetable oils were analysed within 1–2 wk of purchase. Seven of the samples of vegetable oil were obtained in 1974 and were analysed by gas–liquid chromatography (GLC) using an electrolytic conductivity detector (with a limit of detection of about 10 ppb NDMA or NDEA). The remaining samples were purchased in 1980 and GLC analysis was carried out using a thermal energy analyser (TEA). The detection limit of this system was 0·2 ppb for NDMA and NDEA and 0·5 ppb for *N*-nitrosomorpholine (NMOR), *N*-nitrosobutylamine, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine. No nitrosamines were detected in any of the samples of lard or vegetable oils (which included soya-bean, corn, sunflower-seed, olive, groundnut and mixed vegetable oils). One of the butter samples contained 1 ppb NMOR and five samples of margarine contained traces of NMOR and/or NDMA at levels ranging from 1·7 to 3·8 and 0·2 to 0·3 ppb, respectively. The identity of NDMA and/or NMOR in two samples was verified by high-pressure liquid chromatography-TEA.

The authors attempted to determine the source of the contamination by analysing various ingredients of margarine, as well as boiler-water additives, boiler water and steam condensates, all obtained from three margarine factories. (Amine-containing boiler-water additives are commonly used in food-processing plants.) At the same time fresh samples of margarine were obtained from each plant but all of these were found to contain either no nitrosamines or only very low levels (0·2–0·3 ppb) of NDMA or NDEA. No nitrosamines were detected in the boiler waters or the steam condensates, but two of the boiler-water additives (each from a different factory) contained traces of nitrosamines—4·6 ppb NMOR in one, and 1 ppb each of NMOR and NDEA in the other. However these additives were used at very low concentrations (10 lb additive/2 × $10^6$ lb steam). It is not reported that any nitrosamines were detected in the margarine ingredients examined.

The analysis of the margarine ingredients and boiler-water additives was carried out a year after the analysis of the margarines that contained nitrosamines. Different types of boiler-water additive had been used in the past. However, the source of the contamination of the earlier samples could not be pinpointed. The reasons for the considerable differences between the results of this study and that carried out in Germany were not clear.

### NDMA formation from $NO_2$ and dimethylamine?

Iqbal, Z. M., Dahl, K. & Epstein, S. S. (1981). Biosynthesis of dimethylnitrosamine in dimethylamine-treated mice after exposure to nitrogen dioxide. *J. natn. Cancer Inst.* **67,** 137.

Chaudhari, A. & Dutta, S. (1981). Possible formation of nitrosamine in guinea pigs following exposure to nitrogen dioxide and dimethylamine. *J. Toxicol. envir. Hlth* **7,** 753.

The results of a number of studies have indicated that volatile *N*-nitrosamines may be formed *in vivo* by the nitrosation of secondary and tertiary amines by nitrite, which may be ingested as a food additive or formed by bacterial reduction of nitrate in the oral cavity (Fine, *IARC Scient. Publ.* no. 19, 1978, p. 267; Walters *et al. Fd Cosmet. Toxicol.* 1979, **17,** 473). Nitrosation of amines by nitrogen oxides has been demonstrated in liquid and gaseous systems (Bretschneider & Matz, *IARC Scient. Publ.* no. 14, 1976, p. 395; Challis *et al. IARC Scient. Publ.* no. 19, 1978, p. 127; Gehlert & Rolle, *Experientia* 1977, **33,** 579). This has raised the question of whether *N*-nitrosamines can be formed *in vivo* as a result of exposure to $NO_2$, a common atmospheric pollutant. Iqbal *et al.* (*Science, N.Y.* 1980, **207,** 1475) demonstrated the *in vivo* nitrosation by $NO_2$ of morpholine given to mice by gavage. Using similar methods in the study cited above they have now investigated the synthesis *in vivo* of *N*-nitrosodimethylamine (NDMA) by mice treated with dimethylamine (DMA) and exposed to $NO_2$.

Groups of three or four male ICR mice were given 2 mg DMA in 0·25 ml distilled water and then put into chambers in which they were exposed to 41·5 ppm $NO_2$ for 0·5, 1, 2, 3 or 4 hr or to 0·04, 0·1, 0·75, 3, 8, 15, 25 or 44·5 ppm $NO_2$ for 2 hr. Groups of control mice were given no DMA but were exposed to $NO_2$ for the same periods or at the same concentrations as the treated animals. Further controls were treated with DMA or distilled water and exposed to air for times identical to those used for $NO_2$. After exposure the mice were individually frozen in liquid nitrogen and blended to a powder for NDMA analysis using gas chromatography–thermal energy analysis.

NDMA formation was both concentration- and time-dependent with respect to $NO_2$ exposure. NDMA yields were significantly increased over control values (5–12 ng/mouse) at $NO_2$ concentrations of 0·1 ppm or above; for concentrations ranging from 8 to 44·5 ppm, NDMA formation increased linearly with $NO_2$ dose, reaching a maximum of 60–70 ng/mouse, an amount equivalent to 0·0034% conversion

of DMA. A similar level of NDMA synthesis was reached after 2-hr exposure to 41·5 ppm $NO_2$, but in mice exposed to this level for 4 hr less than 20 ng NDMA/mouse was detected. Administration of 50–250 mg sodium ascorbate or 10–100 mg ammonium sulphamate after gavage with 2 mg DMA significantly inhibited NDMA formation in mice exposed to 40–45 ppm $NO_2$ for 2 hr. Ammonium sulphamate was most effective, inhibiting NDMA synthesis by 93–97% in comparison with 54–92% inhibition by sodium ascorbate. (Adding sodium ascorbate or ammonium sulphamate to powdered homogenates of mice that had been exposed to 2 mg DMA and 40–45 ppm $NO_2$ for 2 hr did not cause such decreases in NDMA yield.)

Chaudhari & Dutta (cited above) took a rather different approach to investigate the same topic. Having established that the distribution of $^{14}C$-labelled DMA and NDMA was similar in the rat and guinea-pig and that covalent binding of the $^{14}C$ with nucleic acids was similar in the lungs of the two species but was greater in the guinea-pig liver than in the rat liver, they gave a group of four guinea-pigs a single intravenous injection of 10 mg DMA together with 80 $\mu$Ci [$^{14}C$]DMA/kg body weight and then exposed them to 50 ppm $NO_2$ for 4 hr. A similar group was exposed to 90 ppm $NO_2$ for 8 hr, an intraperitoneal injection of 10 mg DMA and 160 $\mu$Ci [$^{14}C$]DMA/kg body weight being given after the first 3 hr of the $NO_2$ exposure. Controls were given [$^{14}C$]DMA but breathed room air. At the end of the exposure periods the animals were killed and the amount of covalent binding of $^{14}C$ to nucleic acids in liver and lung tissue was determined. Exposure to $NO_2$ caused no significant increase in the amount of covalent binding, and thus there was no indication that the combined treatment with DMA and $NO_2$ had resulted in NDMA formation.

[These studies have yielded conflicting results. However the methodology of the two studies differs considerably. Iqbal *et al.* (cited above) appear to have carried out a carefully controlled investigation although the levels of NDMA detected were very low. Further work to verify their findings and if so to determine the site and mechanism of NDMA synthesis would be valuable.]

## Relaxing over coffee

Aeschbacher, H. U. & Chappuis, C. (1981). Nonmutagenicity of urine from coffee drinkers compared with that from cigarette smokers. *Mutation Res.* **89,** 161.

Readers will be aware that the consumption of caffeine-containing drinks has recently been under the toxicological spotlight (*Food Chemical News* 1980, **22** (26), 35; *Cited in F.C.T.* 1981, **19,** 510; *ibid* 1981, **19,** 789; *ibid* 1982, **20,** 137). Unfortunately, most of the studies reported have been on rats. We now report a study in man comparing the mutagenic potential of urine from instant-coffee drinkers with that from cigarette smokers.

An initial experiment involved three groups of volunteers: six non-coffee drinkers, six who ingested 12 g (equivalent to about six cups) of coffee per day for 4 days, and three who ingested 12 g of coffee per day for 4 days and smoked 20–30 cigarettes daily. The volunteers were given a standard diet throughout the experiment and all of the urine passed in the 4-day treatment period was collected. In a second experiment, twelve nonsmokers and six smokers were given 1 litre of water to drink within 2 hr of a standard breakfast. Urine was collected during the 7 hr following breakfast, and no food was given during this period. A week later the procedure was repeated with 1 litre of water containing 12 g of coffee. The smokers consumed 7–18 cigarettes during each 7-hr period. Any potentially mutagenic compounds bound to urine conjugates were released by treating portions of each urine sample with $\beta$-glucuronidase. Urines were fractionated by XAD-2 column chromatography, and the non-polar fractions were tested in the Ames test using *Salmonella typhimurium* strains TA98 and TA100, with and without metabolic activation. It was calculated that the highest concentration of urine fraction used per plate represented an equivalent of 0·8 g coffee/plate. The fractions obtained from nonsmokers were not mutagenic to either strain in either experiment, with or without $\beta$-glucuronidase treatment or metabolic activation. However, those fractions obtained from smokers were mutagenic when metabolically activated in both strains in the first experiment and in TA98 in the second. (The mutagenicity of urine fractions from smokers was not tested in TA100 in the second experiment.) No significant difference was observed in the number of revertants occurring when smokers drank coffee instead of water.

[Coffee has been reported to be mutagenic to TA100 in the absence of metabolic activation (Aeschbacher & Würzner, *Toxicology Lett.* 1980, **5,** 139; Nagao *et al. Mutation Res.* 1979, **68,** 101). The apparent absence of mutagenic potential in urine fractions from non-smoking coffee drinkers in this study seems to indicate that the human metabolic system has some detoxification capability. The results of the second experiment exclude the possibility of a synergistic effect of coffee, since drinking coffee or water did not significantly alter the mutagenic potential of smokers' non-polar urine fractions. The strong association between coffee consumption and smoking is emphasized by the unwillingness of the three smokers in the first experiment to refrain from drinking coffee during the 4-day test period.]

## Anencephalus and water hardness

Bound, J. P., Harvey, P. W., Brookes, D. M. & Sayers, B. McA. (1981). The incidence of anencephalus in the Fylde peninsula 1956–76 and changes in water hardness. *J. Epidem. commun. Hlth* **35,** 102.

There are marked regional and local differences within the UK in the frequency of congenital malformations of the central nervous system and it has been suggested that there may be a relationship between the incidence of such malformations and the softness of the water supply (Crawford *et al. Lancet* 1972, **i,** 988; Lowe *et al. Br. med. J.* 1971, **2,** 357), although it

may be a secondary association. Bound *et al.* (cited above) have investigated whether changes in the incidence of anencephalus in the most populous part of the Fylde peninsula in Lancashire can be correlated with water hardness.

The incidence of anencephalus in babies born in the study area was 3·2/1000 births between 1957 and 1961, but significantly more cases were born to mothers living in the north of the area than in the south. (The area was divided by a line at 360°N, running roughly through the centre of Blackpool.) There was a slight fall in the rate, to 2·5/1000 births, in 1962–1966 and a significant decrease to 1·3/1000 births in 1967–1976. The national levels at that time were 1·7–1·8/1000 births, and the trends in the northwest as a whole did not follow those observed in the Fylde. It therefore seemed likely that a local environmental change occurred in the Fylde in the 1960s. It also appeared that the process generating cases in the north of the region could be different from that in the south, since after 1967 in the north Fylde the decrease in the conception of anencephalic babies was greater during the summer (May–October) than during the winter. However, no such seasonal difference was observed in the south.

During the early part of the study when more cases of anencephalus were born in the north of the Fylde than in the south, the whole of the area had been supplied with soft water. In 1962 the hardness of the summer water began to increase steadily and by 1967, when the significant drop in anencephalus incidence started it was approaching the 'slightly hard' category. A temporary increase in the hardness of water during winter in 1972 and 1973 did not correspond with any further decrease in the incidence of anencephalus.

It therefore seemed unlikely that water softness was a primary factor in the aetiology of anencephalus, although it is possible that the decrease in cases in the north, which occurred mainly among babies conceived during the summer, was associated with the increased hardness of the water during summer and that hard water may mitigate the effects of other aetiological factors. The authors suggest that if hard water does have an effect, a trace element contained in it may be responsible, perhaps having an effect when the annual intake reaches a certain level.

It seems from this study that soft water is not a primary but a secondary factor in the aetiology of anencephalus and that multiple environmental factors are involved, with individually small but cumulative effects.

**Dioxins and dibenzofurans in bottle teats**

Górski, T. (1981). Presence of polychlorinated dibenzo-*p*-dioxins in latex nipples. *Bull. envir. Contam. Toxicol.*, **27**, 68.

Technical grade pentachlorophenol (PCP) is frequently contaminated with toxic by-products, such as polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (*Cited in F.C.T.* 1979, **17**, 405). Five cases of leukaemia in railway workers have been tentatively attributed to these contaminants in PCP used to treat packing cases (*Pesticide & Toxic Chemical News* 1980, **8** (10), 25; *Nature, Lond.* 1980, **283**, 418) and their presence in chlorophenols and phenoxyacetic acids may have caused an increased incidence of soft-tissue sarcomas in Sweden (*Cited in F.C.T.* 1980, **18**, 541). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin appears to be by far the most hazardous member of its class, but a mixture of 1,2,3,6,7,8- and 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (HCDD) has caused liver tumours in rats and mice (*Federal Register* 1980, **45**, 81126). However, no definite teratogenic effects were produced in mice by 2,7-dichloro-, 2,3,7-trichloro-, 1,2,3,4-tetrachloro- or octachlorodibenzo-*p*-dioxins (*Cited in F.C.T.* 1979, **17**, 95). Partly because of the HCDD content of PCP, the EPA has recently proposed to restrict its use (*Federal Register* 1981, **46**, 13020).

Because PCP is widely used in rubber plantations, an investigation has been undertaken in Poland into polychlorinated dibenzo-*p*-dioxins and dibenzofurans in babies' bottle teats made from imported rubber. Two types of teat were cut into 2–3 mm broad pieces and refluxed for a total of 18 hr with *n*-hexane. High-performance gas chromatography revealed no tetra- or pentachlorodibenzo-*p*-dioxins, but 0·6 and 0·03 ppb of the hexa-, 4·1 and 0·1 ppb of the hepta- and 11·2 and 0·8 ppb of the octa-derivatives. Tetra- and pentachlorodibenzofurans were also undetectable, but the two types of teat contained 0·8 and 0·02 ppb of the hexa-, 3·1 and 0·1 ppb of the hepta- and 2·8 and 0·3 ppb of the octa- materials. The qualitative composition and quantitative ratios of the different isomers of these compounds were typical of those in technical PCP. The predominant hexa- and heptachlorodibenzo-*p*-dioxin being the 1,2,3,6,7,8- and 1,2,3,4,6,7,8-isomers respectively. When the nipples were extracted with water for 24 hr at 100°C, none of these compounds were detectable, suggesting that there may be no real hazard to babies. However, the presence of other toxic contaminants such as chlorinated phenoxyphenols in the extracts was not excluded.

[The experimental conditions under which these toxic contaminants were extracted from the rubber were very severe. A more realistic test might have involved the use of a synthetic triglyceride mixture to simulate the fatty components of milk.]

**Aplastic anaemia in children exposed to organophosphates?**

Reeves, J. D., Driggers, D. A. & Kiley, V. A. (1981). Household insecticide associated aplastic anaemia and acute leukaemia in children. *Lancet* **II**, 300.

Several popular household insecticides in the USA contain organophosphorus compounds such as malathion and dichlorvos (DDVP), and carbamates such as propoxur. This report suggests that there may be a connection between heavy exposure to their vapours and the onset of aplastic anaemia and acute lymphoblastic leukaemia in children, albeit a rare occurrence.

Over a period of 8 yr at the David Grant Medical Center in California, seven children aged 4–12 yr were treated for bone-marrow failure shortly after exposure to these insecticides. None of the children had been exposed to other known haematotoxins but each had been exposed to organophosphates on at least one

occasion 1–28 wk before diagnosis. In six cases, this involved preparations containing a mixture of DDVP with propoxur inhaled for periods estimated to be from 2 min, in the case of one child exposed to very dense fumes in a confined space, up to periods of 2 days when homes had been fugimated by the parents. A seventh child, one of two who subsequently died, inhaled fumes of malathion for about 1 hr.

Blood counts were previously normal but following exposure to the insecticides each child showed a moderate to severe pancytopaenia. In six patients this was diagnosed as aplastic anaemia. This was most severe in the child exposed to malathion, who died 6 months later despite intensive steroid- and immunotherapy. One child responded to steroid therapy and relapses of anaemia and/or thrombocytopaenia in two of the other surviving children did not require treatment or were controlled by treatment with steroids. However another child required regular blood transfusions for severe pancytopaenia. Foetal haemoglobin levels were raised in five of the six patients tested on admission and remained high 3–5 yr later in two children.

Acute lymphoblastic leukaemia was diagnosed in the seventh child, an 11-yr-old girl who had inhaled DDVP and propoxur for approximately 24 hr. She died of progressive disease and *Aspergillus* infection 3 yr later. A younger child who had received a similar exposure was in the preleukaemic aplastic phase for 6 months before converting to acute lymphoblastic leukaemia, but she achieved full remission on combination chemotherapy.

This is believed to be the first report of haematotoxicity associated with organophosphate exposure in children. The authors point to some evidence associating occupational exposure to organophosphates and carbamates with haematotoxicity and leukaemia. They conclude that it is not proven but seems likely that the aplastic anaemia and acute lymphoblastic leukaemia present in the children was the result of exposure to the pesticides and recommend extreme caution in the use of such products when children are likely to be exposed.

[Reeves *et al.* (cited above) point to some evidence associating occupational exposure to organophosphates and carbamates with haematotoxicity. However, the majority of the children were exposed to DDVP, which has not been reported to have haematotoxic effects except for the inhibition of plasma and erythrocyte cholinesterase (*Cited in F.C.T.* 1970, **8**, 461; *ibid* 1975, **13**, 285). It would be interesting to know what proportion of the total number of cases of aplastic anaemia and acute lymphoblastic anaemia seen by the authors was represented by these reported cases.]

### Occupational exposure to an aluminium silicate

Musk, A. W., Greville, H. W. & Tribe, A. E. (1980). Pulmonary disease from occupational exposure to an artificial aluminium silicate used for cat litter. *Br. J. ind. Med.* **37**, 367.

There have been several reports associating occupational exposure to mineral dusts with respiratory effects. For example, workers exposed to kaolin, predominantly consisting of hydrated aluminium silicate, developed disabling pneumoconiosis (Lesser *et al. Sth. med. J., Nashville* 1978, **71**, 10) while pulmonary fibrosis has been described following occupational exposure to dusts of aluminium powder (Mitchell *et al. Br. J. ind. Med.* 1961, **18**, 10). In the paper cited above, workers engaged in bagging an artificial crystalline aluminium silicate were studied following a complaint from one of the employees of respiratory and systemic symptoms, including arthritis.

The aluminium silicate, which had no fibrous content, was the kiln-dried residue obtained from the calcining and water extraction of alunite, a hydrated sulphate of aluminium and potassium. Of the 25 subjects known to have been exposed to the dust, 17 agreed to participate in the study. The average age of the volunteers was 29·1 yr, ranging from 15·1 to 52·1 yr. Age correlated with duration of employment and severity of exposure, in that the older subjects stayed in employment longer and were assigned to the more dusty jobs.

The employee who complained of respiratory ailments began to experience shortness of breath and a dry cough within about 8 months of the onset of exposure. Symptoms of arthritis, general malaise and anorexia were also noted. Lung function tests and radiographical examination suggested that pulmonary fibrosis had developed, a hypothesis confirmed by lung biopsy which revealed a diffuse fibrosis with granulomas. There was a clear temporal relationship between the onset and remission of symptoms and exposure to alunite residue in the workplace.

When specifically questioned, six of the 17 subjects participating in the study considered that exposure to the dust had adversely affected their respiratory systems, although no difference in pulmonary function was observed between these six subjects and the other volunteers. However, three of the subjects (including the one who had complained) gave abnormal chest radiographs consistent with pulmonary fibrosis, and were also found to have functional disturbances of the lungs (when compared with the other 14 subjects) as revealed by their transfer factors for carbon monoxide. Since these three employees had experienced a significantly greater mean total exposure than the others, the authors suggest that there may be a relationship between inhalation of the dust and pulmonary fibrosis. The authors point out, however, that the interpretation of this study is hampered by the small size of the exposed population, the possible confounding effect of smoking, the association between age and total exposure and the lack of matched controls.

[The intensity of alunite-residue exposure in this study was estimated on a 1–3 scale on the basis of the type of work involved, the relevant grade then being multiplied by a time factor depending on the exposure period. The actual concentrations of the silicate in the factory had not been measured quantitatively; such measurements would obviously be useful.]

### Cadmium poisoning

De Silva, P. E. & Donnan, M. B. (1981). Chronic cadmium poisoning in a pigment manufacturing plant. *Br. J. ind. Med.* **38**, 76.

Workers occupationally exposed to cadmium (Cd) compounds risk inhalation and alveolar absorption of the respirable fractions of airborne Cd dusts and fumes. In addition, the non-respirable fractions may be swallowed following clearance from the upper respiratory tract. In man, the principal toxic effects resulting from long-term exposure to Cd are renal dysfunction and lung impairment (*WHO Technical Report Series* no. 647, 1980, p. 21), both of which are reported in a recent study on workers in a Cd-pigment manufacturing plant in Australia.

Measurement in July 1977 of time-weighted-average concentrations of Cd in air demonstrated that workers were exposed to at least 1 mg total 'inhalable' Cd dust/$m^3$ air, of which about half was respirable. This represents at least ten times the ACGIH TLV of 0·05 mg/$m^3$ and greatly exceeds the WHO tentative recommendation of 0·01 mg/$m^3$ (WHO *loc. cit.*). Initially, nine present employees (A–I), a manager (J), and two previous employees (K & L) were examined, the majority being seen at the end of 1976. The six men who had worked at the factory for more than 7 yr (G–L) showed signs of chronic cadmium poisoning. G, H, I, K and L were excreting more than 20 $\mu$g Cd/day in their urine. H, I, J, K and L were excreting protein in their urine and G displayed evidence of damage to kidney tubules. G, H, I, J and L showed impaired ability to acidify urine, and G, H, J and L were unable to concentrate urine fully. H and G, who were both fairly heavy smokers (20 cigarettes/day), showed moderate to severe emphysema, and L showed moderate airways obstruction, as did E who had been exposed to cadmium for only 3 yr and, like L, was only a light smoker. Of particular interest was the manager, J, who had been virtually unexposed to Cd for 10 yr prior to assessment but was still excreting a relatively high amount of Cd in his urine (14 $\mu$g/day) and also showed proteinuria.

Immediately after the initial assessment, H was retired and F, G and L transferred to another section of the factory where exposure to Cd was insignificant. E remained in the same job but started wearing an air-supplied respirator while inside the factory. During the following 2 yr blood Cd concentrations of these subjects (E, F, G, H, I) fell although the decrease was much smaller in the second year. Urinary Cd concentrations decreased initially but remained quite high throughout the second year.

A follow-up study on eleven new employees (exposure time between 1·8 and 12 months) indicated that blood cadmium concentrations increase soon after exposure begins, but exposure is not reflected in an increased urine cadmium excretion for about 3 months. The authors suggest that proteinuria is a good indication of renal damage in workers exposed to cadmium and that in people with past exposure to Cd, urinary Cd concentrations of less than 5 $\mu$g/day do not indicate freedom from kidney damage. They considered that respirable insoluble compounds, which accounted for much of the Cd to which these workers were exposed, probably contributed significantly to lung damage and that to regard the non-respirable insoluble compounds as merely 'nuisance' dust is to underestimate their hazard potential.

**Trivalent chromium further incriminated**

Fornace, A. J., Jr, Seres, D. S., Lechner, J. F. & Harris, C. C. (1981). DNA-protein cross-linking by chromium salts. *Chemico-Biol. Interactions* **36,** 345.

It has been suggested, on the basis of the results of *in vitro* studies of metabolism and mutagenicity, that the trivalent form of chromium ($Cr^{III}$) may be ultimately responsible for the carcinogenic effects that have been associated with occupational exposure to chromium salts (*Cited in F.C.T.* 1980, **18,** 102). The production of stable linkages between DNA and protein *in vitro* by $Cr^{III}$ and hexavalent chromium ($Cr^{VI}$) has now been reported.

Cultures of human embryonic lung fibroblasts, human bronchial epithelium and mouse leukaemia cells were used in the study. DNA–protein cross-linking was determined by the alkaline-elution technique. The basis for this technique is that single strands of DNA have been shown to elute through a filter at a rate dependent on their size whereas when the DNA is cross-linked to protein it is retained on the filter, presumably because of binding of the protein to the filter during alkaline elution. DNA, from untreated normal cells, that has been fragmented by exposure to $\gamma$-radiation has an increased elution rate, but this increase is much less marked when DNA that has formed stable cross-links with protein is exposed to the same dose of $\gamma$-radiation. A 'cross-link factor' can be derived by measuring the relative retentions on the filter of cells treated with chromium salts and untreated cells that have or have not been exposed to $\gamma$-radiation.

$Cr^{VI}$ added to whole-cell cultures (in the form of potassium chromate) induced cross-linking in a dose-dependent manner. These cross-links were relatively persistent since no significant change was observed after 12 hr of repair incubation. The level of DNA single-strand breaks was obscured by the cross-linking but an increase in elution rate following proteinase digestion of the cell lysates indicated that $Cr^{VI}$ had induced a low level of single-strand breaks in DNA. However these breaks were rejoined after 4 hr of repair incubation. $Cr^{VI}$ added to isolated nuclei did not induce cross-linking, and when $Cr^{VI}$ was first incubated with a reducing agent its induction of cross-linking in whole-cell cultures was markedly decreased. Conversely, $Cr^{III}$ (in the form of chromic chloride) appeared to have no effect on DNA in whole-cell cultures but did induce cross-linking in isolated nuclei. $Cr^{III}$ also induced cross-links between DNA and protein in solution but $Cr^{VI}$ did not. These data add further support to the hypothesis that $Cr^{VI}$ crosses the cell membrane and is reduced to the trivalent form. Stable DNA–protein cross-links induced by $Cr^{III}$ may affect polymerase activity during DNA replication and repair and this may be a basis for the mutagenic effects of chromium.

**Ethylene oxide and DNA repair**

Pero, R. W., Widegren, B., Högstedt, B. & Mitelman, F. (1981). In vivo and in vitro ethylene oxide exposure of human lymphocytes assessed by chemical stimulation of unscheduled DNA synthesis. *Mutation Res.* **83,** 271.

Ethylene oxide (EO) exposure has been associated with chromosome aberrations in animals and man (*Federal Register* 1978, **43**, 3800; *Pesticide & Toxic Chemical News* 1980, **8** (26), 18) and three cases of leukaemia have been reported in EO-exposed Swedish workers (*Cited in F.C.T.* 1979, **17**, 686). Sister-chromatid exchange was increased in the lymphocytes of individuals exposed tc a maximum of 36 ppm EO in a hospital sterilization facility (Garry *et al. Envir. Mutagen.* 1979, **1**, 375). That EO concentrations of only 0·5–1 ppm may also affect the blood cells has now been demonstrated.

The study group consisted of four medical equipment sterilizers and one laboratory technician, exposed to 5–10 ppm EO for a total of 1 hr/day, and 12 packers, exposed to 0·5–1 ppm for 8 hr/day, 5 days/wk. All were female and had worked in the factory for 0·8–8 yr. Eleven female assembly-line workers from another part of the factory with no exposure to EO were used as controls. Unscheduled DNA synthesis (UDS) induced by *N*-acetoxy-2-acetylaminofluorene (AC-AAF) in peripheral lymphocytes was significantly reduced in the packers, and chromosome aberrations (breaks and/or gaps) were significantly increased in both exposed groups. In controls from the present study and those from earlier studies UDS was directly correlated with age and/or with the incidence of chromosome aberrations, whereas in exposed individuals UDS was negatively correlated with duration of exposure and with the number of chromosome breaks, indicating an inhibition of DNA-repair capacity by EO. When EO levels in the factory were subsequently reduced to a maximum of 0·5 ppm, after 9 months UDS had returned to normal.

Leucocytes from whole blood exposed *in vitro* to 0·5–100 mM-EO for 1 hr showed a linear increase in UDS up to 5 mM, after which a reduction occurred. When isolated lymphocytes and leucocytes were exposed to the same concentrations, the maximal level of UDS occurred at only 1–2 mM. Autoradiographic studies on lymphocytes confirmed that UDS was inhibited above 2 mM, whether estimated at 24 or 122 hr. Further studies on lymphocytes indicated that the inhibition of UDS was related to cytotoxicity. Even at a stimulatory dose of 1 mM, although there were only 13% more dead cells than in a control culture after 24 hr, after a further day 70% of the cells were dead, compared with only 11% of control cells. When lymphocytes were exposed to 0·1–1·0 mM-EO for 18 hr followed by 10 μM AC-AAF for 1 hr, only 5–15% were dead after a further hour. However, after a further 18 hr more than 40% had died at all concentrations, suggesting that even at 0·1 mM-EO the lymphocytes were sensitized to subsequent exposure to cytotoxic agents such as AC-AAF. UDS was unaltered in surviving cells at 0·1–0·6 mM, but was reduced at 0·8 or 1 mM.

It is calculated that daily exposure to 0·5–1 ppm EO, as in the packers, would lead to a maximum tissue concentration of only 0·1 μM-EO. In contrast, in the *in vitro* studies effects on UDS were detected only in the mM range, suggesting that repeated exposure must have a pronounced cumulative effect. The correlation between years of exposure and UDS inhibition supported this hypothesis. Since the packers also tended to show a negative correlation between leucocyte count and induced UDS, it is suggested that EO first caused a leucopenia in which surviving cells had a reduced UDS. This was followed by a leucocytosis to replace the dead cells, which resulted in a mixing of cells with normal UDS and inhibited UDS capacities. The final result would be an overall inhibited UDS.

**Reproductive trouble for 2-methoxyethanol**

Nagano, K., Nakayama, E., Oobayashi, H., Yamada, T., Adachi, H., Nishizawa, T., Ozawa, H., Nakaichi, M., Okuda, H., Minami, K. & Yamazaki, K. (1981). Embryotoxic effects of ethylene glycol monomethyl ether in mice. *Toxicology* **20**, 335.

Industrial limits for exposure to the glycolethers may be on the move. The present TLV of 25 ppm set for 2-methoxyethanol (ethylene glycol monomethyl ether) was based on a limited pre-war epidemiological study in which exposed workers were found to develop encephalopathy and anaemia. There has recently been a renewed interest in the reproductive toxicology of the glycol ethers as a class. Teratogenic effects have been reported in rats, mice and rabbits exposed to ethoxyethanol and testes damage occurred in rats and rabbits exposed to atmospheres containing 2-methoxyethanol (*Pesticide & Toxic Chemical News* 1981, **9** (17), 8). The mouse study cited above suggests that 2-methoxyethanol may also have teratogenic effects.

Groups of 21–24 pregnant ICR mice were given daily doses of 0, 31·25, 62·5, 125, 250, 500 or 1000 mg 2-methoxyethanol/kg body weight by gavage on days 7 to 14 of gestation and then killed on day 18. Increased foetal deaths occurred at maternal doses of 250 mg/kg and above. All the litters were dead in the 1000 mg/kg group and there was only one live foetus among the 500 mg/kg group. The first indication of a reduction in foetal body weight was observed at 125 mg/kg. A high incidence of gross abnormalities (exencephaly, abnormal fingers and umbilical hernia) was found at 250 mg/kg. There was a dose-related increase in the incidence of malformations at doses of 62·5 mg/kg and above. Skeletal variations such as cervical ribs, asymmetry of the sternebrae, and bificurated or split cervical vertebrae were also increased at all doses in a dose-related manner. There was evidence of a retardation in bone ossification in all treatment groups. Examination of the maternal animals—limited to clinical examination, measurement of weight gain and leucocyte sampling—indicated that doses of 250 mg/kg and above were toxic, reducing maternal weight gains. Decreased leucocyte count was limited to the dams of the highest dose group.

**Does DNA binding reflect carcinogenic potency?**

Pereira, M. A., Lin, L.-H. & Chang, L. W. (1981). Dose-dependency of 2-acetylaminofluorene binding to liver DNA and hemoglobin in mice and rats. *Toxic. appl. Pharmac.* **60**, 472.

Many chemical carcinogens or their active metabolites have been shown to bind to DNA. To determine whether the extent of such binding is correlated

with carcinogenic potency, a study of its dose-dependency has been undertaken with 2-acetylaminofluorene (2-AAF) for which the relationship between dose and liver tumour induction has been established in mice (Littlefield *et al. J. envir. Path. Toxicol.* 1979, **3** (3), 17) and rats (Albert *et al. Cancer Res.* 1972, **32**, 2172). The dose-dependency of haemoglobin binding was also studied, because this has been proposed as a systemic dose monitor of DNA binding in target organs.

Rats and mice given [9-$^{14}$C]2-AAF by gavage at doses of 1, 10 or 100 μmol/kg body weight were killed 24 hr later, and DNA–2-AAF adducts in the liver were determined chromatographically after enzymatic degradation of DNA to deoxyribonucleosides. The elution profile from rat liver contained four major peaks, of which one was identified as the DNA adduct of *N*-(deoxyguanosine-8-yl)-*N*-AAF and another (the major peak) appeared to be the adduct of deacetylated 2-AAF. The relative contribution of these four peaks to the total acylation of DNA was independent of dose, and together they contained more than 90% of the radioactivity. Binding in both mice and rats was a linear function of dose, and in rats was about sevenfold greater than in mice. The liver DNA of female mice bound about four times as much $^{14}$C as that of male mice, whereas in rats no sex difference was apparent. In the haemoglobin of treated rats, more than 95% of the radioactivity represented 2-AAF metabolites covalently bound to amino acids. In both species binding was again directly dependent on dose (0·1, 1, 10 or 100 μmol 2-AAF/kg body weight), and in rats was 2·5 times greater than in mice, but there were no apparent sex differences. It was calculated that rats and mice bound 0·07% and 0·03%, respectively, of an oral dose of 2-AAF to haemoglobin.

The findings indicated that over the dose range studied the formation and DNA binding in the liver of the active metabolites of 2-AAF was linearly related to dose, and that haemoglobin binding could be used to predict DNA in the liver. However, in the long-term rat and mouse studies (Albert *et al. loc. cit.*; Littlefield *et al. loc. cit.*) the potency of 2-AAF was 246-fold greater in rat than in mouse liver, a far greater difference than the sevenfold discrepancy in DNA binding. It is suggested that the extent of DNA binding may correlate better with initiation than with carcinogenic potency, and that the latter depends on the subsequent influence of other factors. Moreover, although female mice and male rats are more susceptible to 2-AAF-induced hepatocarcinogenesis than male mice and female rats, these differences were reflected only in the higher binding to mouse-liver DNA in females than in males. In rats the difference in susceptibility has been associated with testosterone (Toh, *Adv. Cancer Res.* 1973, **18**, 155) and in this case would appear to depend not on relative DNA binding but on promotion by sex steroids.

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# MEETING ANNOUNCEMENTS

## IDENTIFICATION OF ASBESTOS

A McCrone Research Institute course on the use of the polarizing microscope to identify different types of asbestos will be held at the Holiday Inn, Swiss Cottage, London NW3 on 5 and 6 October 1982. Special emphasis will be placed on the types of asbestos that have been or still are commercially available in Europe. The course will be followed by a seminar on the membrane filter technique for monitoring and counting asbestos fibres, which will take place on 7 February at the same venue. Further details and registration forms on both of these courses are available from The Registrar, McCrone Research Institute Ltd, 2 McCrone Mews, Belsize Lane, London NW3 5BG (tel. 01-435 2282/3/4, Telex 8952387 McResearch).

## CHEMICALS IN THE ENVIRONMENT

A symposium on chemicals in the environment has been organized by the Danish Ministry of Environment, Agency of Environmental Protection. The symposium will concentrate on reviews and evaluations of the methods in use or being developed for the toxicity testing of chemicals, and the administration processes used for hazard assessment and hazard ranking of chemicals. The symposium will take place at the Technical University of Denmark, Lyngby, Copenhagen on 18–20 October 1982. The language of the symposium will be English. Inquiries should be addressed to DIS Congress Service, 48 Linde Allé, DK-2720 Vanløse/Copenhagen, Denmark (Telex 15476 Discon DK).

## ENVIRONMENTAL EFFECTS ON MAN

Topics to be discussed at a meeting on the influence of the environment on man include toxicological problems in the urban environment and the toxicology of cosmetics. The meeting has been organized by the International Society for Research on Civilization Diseases and Environment and will be held on 17–20 November 1982 in Vienna. Details can be obtained from Hofrat DDr Maruna, Strahlenabteilung der I und II, Univ.-Frauenklinik, Spitalgasse 23, 1-1097 Vienna IX, Austria or from F. Hendrickx, rue E. Bouillot 61, Box 11, B-1060, Brussels, Belgium.

## INORGANIC AND NUTRITIONAL ASPECTS OF CANCER

A conference entitled "Inorganic and Nutritional Aspects of Cancer and Other Diseases" organized by The International Association of Bioinorganic Scientists is to be held at La Jolla Village Inn, La Jolla CA on 10–13 November 1982. Further information may be obtained from Dr G. N. Schrauzer, Department of Chemistry, University of California, San Diego, Revelle College, La Jolla, CA 92093.

## USING BACTERIA FOR TOXICITY SCREENING

The First International Symposium on Toxicity Testing Using Bacteria is to be held at the National Water Research Institute, Burlington, Ontario on 17–19 May 1983. The proposed sessions for the symposium are on oxygen uptake inhibition, growth inhibition, commercial toximeters using ATP, morphological responses, inhibition of colony formation on algae, dehydrogenase inhibition, mineralization process inhibition and substrate utilization inhibition. Abstracts have been invited and must be submitted by 20 August 1982. Further details are obtainable from B. J. Dutka, Analytical Methods Division or D. Liu, Environmental Contaminants Division, The National Water Research Institute, Canada Centre for Inland Waters, PO Box 5050, Burlington, Ontario, Canada L7R 4AS.

## OCCUPATIONAL MEDICINE COURSE

A 6-week course for physicians who are interested in the practice of occupational medicine will be held by the Division of Occupational Medicine and Extended Programs in Medical Education of the University of California School of Medicine at San Francisco, CA during 1982/83. The course will comprise three separate 2-week sessions: 23 August–3 September 1982, 14–25 March 1983 and 5–16 September 1983. Further details may be obtained from Extended Programs in Medical Education, University of California School of Medicine, Room 569-U, Third and Parnassus Avenue, San Francisco, CA 94143 (tel (415) 666–4251).

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# CORRIGENDUM

*Volume 20 (1982)*

p. 131, line 31 (R.H. col.): *For* Technology *read* Toxicology.

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# FORTHCOMING PAPERS

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

The metabolic disposition of $^{14}$C-labelled carmoisine in the rat after oral intravenous administration. By C. L. Galli, M. Marinovich and L. G. Costa.

Short-term toxicity study of carnauba wax in rats. By I. R. Rowland, K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

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

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

Nitrosamine levels in human blood, urine and gastric aspirate following ingestion of foods containing potential nitrosamine precursors and preformed nitrosamines. By L. Lakritz, R. A. Gates, A. M. Gugger and A. E. Wasserman.

A comparison of the effects of lactose and of two chemically modified waxy maize starches on mineral metabolism in the rat. By A. Hodgkinson, D. Davis, J. Fourman, W. G. Robertson and F. J. C. Roe.

Mutagenicity testing in the *Salmonella typhimurium* assay of phenolic compounds and phenolic fractions obtained from smokehouse smoke condensates. By B. L. Pool and P. Z. Lin.

Dose-response studies in carcinogenesis by nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine in rats and the effect of deuterium. By W. Lijinsky, M. D. Reuber, T. S. Davies, J. E. Saavedra and C. W. Riggs.

The fate of *N*-nitrosodiethanolamine after oral and topical administration to rats. By E. J. Lethco, W. C. Wallace and E. Brouwer.

Effect of cyclopropenoid fatty acids on the hepatic microsomal mixed-function-oxidase system and aflatoxin metabolism in rabbits. By T. A. Eisele, P. M. Loveland, D. L. Kruk, T. R. Meyers, J. E. Nixon and R. O. Sinnhuber.

Problems associated with the use of cycloheximide to distinguish between animal drug residues bound to protein and those incorporated into protein. By P. G. Wislocki, K. M. Fiorentini, R. F. Alvaro, A. Y. H. Lu and F. J. Wolf.

Comparative rates of elimination of some individual polychlorinated biphenyls from the blood of PCB-poisoned patients in Taiwan. By P. H. Chen, M. L. Luo, C. K. Wong and C. J. Chen.

Evaluation, using *Salmonella typhimurium*, of the mutagenicity of seven chemicals found in cosmetics. By M. J. Prival, A. T. Sheldon, Jr and D. Popkin.

The morphogenesis of renal cortical tumours in rats fed 2% trisodium nitrilotriacetate monohydrate. By C. L. Alden and R. L. Kanerva.

Alterations of renal tissue structure during a 30-day gavage study with nitrilotriacetate. By J. A. Merski.

Unaffected blood-boron levels in newborn infants treated with a boric acid ointment. By B. Friis-Hansen, B. Aggerbeck and J. Aas Jansen.

Tartrazine-induced chromosomal aberrations in mammalian cells. By R. M. Patterson and J. S. Butler. (Short Paper)

Qualitative detection of *N*-nitrosodiethanolamine in cosmetic products. By M. Tunick, H. S. Veale and G. W. Harrington. (Short Paper)

*Contents continued*]



## *Aims and Scope*

The Journal publishes original papers and reviews relating to the interests of the British Industrial Biological Research Association. This is a wide-ranging field covering all aspects of toxicology but with particular reference to food. The Journal aims to be informative to all who generate or make use of toxicological data.

# *Instructions to Authors*

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

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

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

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

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

e.g. Guppy, L., Lowes, N. R. & Walker, M. J. A. (1981). Effect of a proprietary rubefacient "Tiger Balm" on rabbit skin. *Fd Chem. Toxic.* **20**, 89.

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

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

When reference to a paper is first made in the text the names of all authors should be given unless there are five or more, in which case the names of the first three should be given followed by the words *et al.* In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963; Malinow, McNulty, McLaughlin *et al.* 1981); (McLaughlin *et al.* 1963; Malinow *et al.* 1981).

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

e.g. 1943a, 1943b or (1943a,b).

**Footnotes.** These, as distinct from literature references, should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

**Illustrations and Diagrams.** These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on a *separate* sheet. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

▲ △ ■ □ ● ○ ○ × ◇

Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for colour separation is the responsibility of the author.

**Tables and Figures.** These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

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

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

**Page Proofs.** These will be sent to the first-named author for correction.

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