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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

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

Fd Cosmet. Toxicol. Vol. 14, p. ii. Pergamon Press 1976. Printed in Great Britain

FORTHCOMING PAPERS

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

Failure of FD & C Red No. 2 to produce dominant lethal effects in the mouse. By D. W. Arnold, G. L. Kennedy, Jr., M. L. Keplinger and J. C. Calandra.

Dimethylnitrosamine and nitrosopyrrolidine in fumes produced during the frying of bacon. By N. P. Sen, S. Seaman and W. F. Miles.

Studies on aflatoxin B_1 toxicity in female rats pretreated with an oral contraceptive agent. By M. U. K. Mgbodile and M. Holscher.

Effects of prenatal administration of citrinin and viriditoxin to mice. By R. D. Hood, A. W. Hayes and J. G. Scammell.

The distribution and excretion of hexachlorophene in rats of different ages. By K. Bjondahl and B. Isomaa.

The metabolism of the antibacterial agent bronopol (2-bromo-2-nitropropane-1,3-diol) given orally to rats and dogs. By D. H. Moore, L. F. Chasseaud, J. D. Lewis, P. C. Risdall and E. L. Crampton.

The percutaneous absorption and disposition of the antibacterial agent bronopol in rats and rabbits. By D. H. Moore, L. F. Chasseaud, D. Bucke and P. C. Risdall.

Formation of dimethylnitrosamine from pesticides carrying methylated tertiary amino groups in the presence of nitrite at pH 3. By G. Egert and H. Greim. (Short Paper)

Inhibition of sensitization reactions induced by certain aldehydes. By D. L. J. Opdyke. (Short Paper)

Fd Cosmet. Toxicol. Vol. 14, pp. 85–93. Pergamon Press 1976. Printed in Great Britain

Research Section

CARRAGEENAN: THE EFFECT OF MOLECULAR WEIGHT AND POLYMER TYPE ON ITS UPTAKE, EXCRETION AND DEGRADATION IN ANIMALS

K. A. PITTMAN, L. GOLBERG and F. COULSTON

Institute of Comparative and Human Toxicology, Center of Experimental Pathology and Toxicology, Albany Medical College, Albany, New York 12208, USA

(*Received 17 May 1975*)

Abstract—A variety of ι-, κ- and λ-carrageenans was given to guinea-pigs, monkeys and rats, either in the drinking-water, by gavage or in the diet. Faecal and liver samples were examined qualitatively by gel electrophoresis, to determine any changes in the apparent molecular weight of carrageenans after administration. Quantitative measurements of carrageenans were carried out on samples of liver and urine. That there was little or no absorption of carrageenans of high molecular weight was evidenced by the absence of carrageenan from the livers of guinea-pigs or rats or from the urine of guinea-pigs or monkeys. By contrast, substantial amounts of carrageenan were found in the livers of guinea-pigs and rats given low-molecular-weight carrageenans ($M_n \leqslant 40{,}000$). Intermediate amounts of carrageenan were found in livers of animals given carrageenans ranging in M_n between 40,000 and 150,000. Urinary excretion of carrageenan was limited to low-molecular-weight material ($M_n \leqslant 20{,}000$). Qualitative and quantitative evidence indicated that there was an upper limit to the size of carrageenan molecules absorbed, but estimates of this upper limit ranged from 10,000 to 85,000 depending upon the analytical approach. Absorption of carrageenan from the drinking-water may differ qualitatively from absorption from the diet. Analysis of faecal samples by gel electrophoresis showed that degradation of high-molecular-weight carrageenan had occurred, either in the gut or in the faeces.

INTRODUCTION

Carrageenan is the class name given to a variety of sulphated polysaccharides derived from various species of red algae. There are three main types: ι, κ and λ. Both ι- and κ-carrageenan contain *O*-3-substituted *O*-4-sulphate-β-D-galactopyranosyl units, while λ-carrageenan contains *O*-3-substituted *O*-2-sulphate-β-D-galactopyranosyl units. In alternation with the above moieties, ι-carrageenan contains *O*-4-substituted *O*-2-sulphate-3,6-anhydro-α-D-galactopyranosyl units. κ-carrageenan contains *O*-4-substituted 3,6-anhydro-α-D-galactopyranosyl units and λ-carrageenan contains *O*-4-substituted *O*-2,6-disulphate-α-D-galactopyranosyl units (Stancioff & Stanley, 1969). An otherwise perfect alternating sequence in each of these polymers is masked somewhat by occasional modifications in the placement and number of sulphate groups or, for ι- and κ-carrageenans, by the absence of the 3,6-anhydro- linkage. In solution and in the solid state, ι- and κ-carrageenans form double, right-hand helices, whereas λ-carrageenan forms a zig-zag ribbon (Rees, 1972; Stancioff and Stanley, 1969). Freshly isolated carrageenans range in average molecular weight from $0{\cdot}25 \times 10^6$ to $0{\cdot}75 \times 10^6$, depending upon extraction conditions (Towle, 1973), and the preparations are polydisperse.

Carrageenans (Irish moss) have been used medicinally for centuries. Currently, a degraded ι-carrageenan (hereafter referred to as C16) is marketed in Europe as an anti-ulcer preparation, and high-molecular-weight carrageenans (i.e. those isolated with minimal hydrolytic or other chemical degradation) are used in a wide variety of prepared foods as texturizing and suspending agents. Carrageenans are non-nutritive polysaccharides (Hawkins & Yaphe, 1965), the bulk of which passes through the gut unabsorbed (Dewar & Maddy, 1970; Hawkins & Yaphe, 1965). Several reports have appeared in recent years implicating carrageenans in the formation of ulcers and the production of metaplastic changes in the gastrointestinal tract of animals (Abraham, Fabian, Golberg & Coulston, 1974; Benitz, Golberg & Coulston, 1973; Fabian, Abraham, Coulston & Golberg, 1973; Grasso, Sharratt, Carpanini & Gangolli, 1973; Marcus & Watt, 1969; Sharratt, Grasso, Carpanini & Gangolli, 1970; Watt & Marcus, 1969). Marcus & Watt (1969) and Watt & Marcus (1969, 1970a,b) first described the occurrence of caecal and colonic ulcers caused by undegraded carrageenans of unspecified origins or by C16 in the guinea-pig, rabbit, rat and mouse. The most severe lesions occurred in the guinea-pig and rabbit and the effect was dose-related. Most of the carrageenans used were of the ι-type from *Eucheuma spinosum* (such as C16) and were administered in the drinking-water. The effect was first seen to occur in the caecum, followed by ulceration of the colon with higher doses or longer times of administration. Ulceration in the guinea-pig by orally administered C16 was reported also by Sharratt *et al.* (1970). Grasso *et al.* (1973) claimed that undegraded or degraded carrageenans produced ulcers in guinea-pigs and rabbits but not in rats, hamsters,

squirrel monkeys or ferrets, and attributed the difference to the herbivorous character of the affected species. Others have reported that C16 caused ulcers in rhesus monkeys, whereas undegraded κ,λ-carrageenans did not (Benitz *et al.* 1973), and that squamous metaplasia occurred at the anorectal junction of rats given C16 (Fabian *et al.* 1973).

In most of the above studies, large numbers of macrophages containing metachromatic granules were found at the sites where pathological changes occurred. Abraham *et al.* (1974) have reported that carrageenan is taken up and stored by lysosomes in the macrophages, and have suggested that this process may play an intimate role in the development of ulceration. The presence of metachromatic material has been observed in Kupffer cells after the administration of C16 but not after the administration of undegraded κ,λ-carrageenan to rhesus monkeys (Abraham, Golberg & Coulston, 1972). The excretion of only small amounts of carrageenan in the urine of baboons given large doses of C16 was taken as evidence that little of this material was taken up from the gastro-intestinal tract, but analyses of liver or other organs were not performed (Beattie, Blakemore, Dewar & Warwick, 1970). The presence of carrageenan-like material in amounts up to 1 mg/g in various organs of rats and guinea-pigs (Grasso, Gangolli, Butterworth & Wright, 1975) indicated that substantial amounts of carrageenan had been absorbed. Evidence that there was little or no storage or excretion of carrageenan-like material when undegraded, as compared to degraded, carrageenan was administered (Abraham *et al.* 1972; Anderson & Soman, 1966) and the suggestion that uptake and ulceration were related (Abraham *et al.* 1974) raised the possibility that the molecular weight or even the type of carrageenan chain might influence its ulcerogenic potency. The analyses reported below were carried out to explore these possibilities. They were performed in conjunction with several different morphological studies, which will be reported elsewhere. The analytical results are gathered into a single communication to facilitate comparisons.

EXPERIMENTAL

Carrageenans. Various preparations of carrageenan from the seaweeds *Chondrus crispus* and *E. spinosum* were provided by Marine Colloids, Inc. (Maine), Stauffer Chemical, Inc. (Ohio) and Laboratoires Glaxo (Paris). Different manufacturing processes had been used in the extraction and purification of these polysaccharides. In particular, the use of alkali in the preparation of κ,λ-carrageenans resulted in the desulphation of some of the 2,6-disulphate moieties in λ-carrageenan to form 3,6-anhydro-2-sulphate moieties, i.e. θ-carrageenan. Other effects of manufacturing may result in the inclusion or exclusion of water or various counter-ions. Fractionation of κ,λ-carrageenan to prepare purified κ- and λ-carrageenans from *C. crispus* and degradation of κ-, λ- and ι-carrageenans by mild acid hydrolysis to fractions of lower molecular weight were carried out by Marine Colloids, Inc., as were all determinations of the numerical mean weight (M_n) of the various preparations and of the molecular-weight distributions within various preparations. The preparations used in the studies reported here are listed in Table 1, along with some of their properties. They were used as received.

Table 1. *Types and relative molecular sizes of carrageenans used*

Sample no.	Source	Description (% and type)	$[\eta]$*	M_n†
1	*C. crispus*‡	71 κ, 29 λ	11·1	214.000
2		81 κ, 19 λ	9·9	200.000
3		88 κ, 12 λ	8·0	186.000
4	*E. spinosum*§	100 ι	0·113	5000
5			0·285	8700
6			0·685	21.000
7			1·62	39.000
8			4·19	88.000
9			5·34	107.000
10			7·51	145.000
11	*C. crispus*§	100 κ	0·177	8500
12			1·45	51.500
13			12·0	314.000
14		100 λ	0·503	20.800
15			2·24	74.800
16			10·2	275.000
17		70 κ, 30 λ	—	185.000
18	*E. spinosum*‖	100 ι	0·35	9900

* Intrinsic viscosity of Na^+ salt in dl/g (data supplied by Marine Colloids, Inc.).
† Number average molecular weight by gel electrophoresis (data supplied by Marine Colloids, Inc.).
‡ Stauffer Chemicals, Inc.
§ Marine Colloids, Inc.
‖ Laboratoires Glaxo.

Animals and protocols. Young adult Sprague–Dawley rats of both sexes were divided into groups of three and given either distilled water or one of the carrageenans numbered 5–10 (Table 1) in distilled water in a dose of 500 mg/kg by gavage, daily, for 9 months. At the end of this time, animals were killed and the livers were retained for analysis.

In another experiment weanling Sprague–Dawley rats of both sexes were divided into groups and fed control diet (Wayne Lab Blox, Allied Mills, Inc.,) or a diet containing 5% of one of the carrageenans numbered 1, 2 or 3 (Table 1). After 13 wk, five animals of each group and sex were killed and samples of liver were analysed for carrageenan. Overnight faecal samples were also collected for analysis.

Female Camm–Hartley guinea-pigs (Camm Research Inc.,) were divided into groups and fed control diet (Wayne Guinea Pig Diet) or a diet containing 2% of one of the carrageenans numbered 4–10 (Table 1) for a period of 7–10 wk, after which they were killed and samples of liver from each of the six animals/group were obtained for analysis. In two later experiments, other groups of female guinea-pigs were given a carrageenan either from numbers 4–10 or from numbers 11–16 (Table 1) as 1% solutions in their drinking-water for 2–3 wk. At the end of the first of these latter experiments, samples of liver, faeces and bladder urine were analysed; at the end of the second experiment samples of liver and faeces were examined.

Rhesus monkeys (*Macaca mulatta*) of both sexes were given carrageenan no. 17 daily in doses of 500, 200 or 50 mg/kg by stomach tube. Overnight collec-

tions of faeces were obtained after 11 months. Overnight collections of urine were obtained from two of the monkeys on the high dose after 15 months. Two female rhesus monkeys were each given 1 g (about 0·2 g/kg) of carrageenan no. 18 by stomach tube and complete first-day and second-day urine collections were made.

Isolation of carrageenan from biological materials. Carrageenan was isolated from liver and kidney by the method of Gangolli, Wright & Grasso (1973) with the following modifications: (1) 1-g tissue samples were defatted by grinding them twice in 15 ml acetone in a Potter-Elvehjem tissue homogenizer (Bel-Art Products,), since two defatting steps proved necessary for adequate removal of lipid; (2) after removal of the acetone, the samples were transferred to conical homogenizers (Kontes Glass K885350), using a total of 10 ml 0·1 M-acetate buffer (Na salt), pH 6·0, containing 5 mM-EDTA but no cysteine; when the samples had been homogenized again, 0·1 ml of an aqueous solution of 10% (w/v) cysteine hydrochloride was added at the same time as papain, to provide a maximum of reducing elements at the beginning of the papain digestion; (3) after completion of the trypsin digestion, which follows papain digestion, the samples were transferred to 15-ml centrifuge tubes and centrifuged at 12,000 **g** for 1 hr at 30–35°C to remove material that interfered with the subsequent colorimetric determination of carrageenan; (4) the concentration of the cetylpyridinium chloride solution was increased from 1 to 5%.

After completion of the isolation procedure, the samples were taken up in 0·1 ml water and sub-samples were taken for electrophoretic and/or colorimetric determination of carrageenan. Carrageenan was extracted from faeces by air-drying each sample, grinding it in a mortar and extracting with 50 ml water/g dried material for 2 hr in a boiling-water bath. The samples were centrifuged at 12,000 **g** for 0·5 hr and the supernatants were carefully removed and diluted for colorimetric analysis. Carrageenan in the urine was analysed directly.

Qualitative analysis. Electrophoresis was carried out on rehydratable agarose-gel plates (MCI Biomedical, Rockland, Maine) by the procedure developed by Stanley & Renn (1974). The developed plates were stained with toluidine blue O (Stanley & Renn, 1974) and the mobility of any metachromatic bands was measured in relation to that of the red component of the dye marker (Gelman Co. Cat. 51250). Since the relative mobilities of isolated carrageenans tended to vary according to the medium from which they were isolated, standard samples of the carrageenan in question were always added to control samples, carried through the isolation procedure and run on the same agarose-gel plate as the test samples. Duplicate samples run on the same plate always gave identical results.

Quantitative analysis. Method A: Strips of equal length and width were cut from each channel of the developed, dried gel electropherogram sheets. The strips were folded lengthwise to prevent adherence of the gel surface to test-tube walls and were extracted with 2 ml 6 N-HCl. When all traces of colour had been removed from a strip, 1·4 ml 10 N-NaOH was added and the tube was shaken until all yellow colour had disappeared, after which 2·6 ml 6 N-HCl and 4 ml butanol were added and the tube was shaken again. The absorbance of the butanol phase was read at 630 nm against a reagent blank. Method B: Appropriate volumes of the reconstituted pellet from tissue extraction, of urine or of faecal extract were combined with 1-ml volumes of 0·0075% toluidine blue O, and each mixture was made up to a final volume of 10 ml and read at 630 nm against appropriate blanks (Beattie *et al.* 1970). For either method, control samples of tissue, urine or faeces alone and similar control samples to which known amounts of carrageenan had been added were carried through the entire isolation, electrophoresis and quantitation procedures in company with experimental samples. In this manner, an 'extracted' standard curve was available with each set of experimental samples to reflect losses and other sources of error.

RESULTS

Methodology

Several modifications of the isolation procedure were attempted in the hope of reducing the analysis time, which was greater than 1 wk. Unfortunately, no modification that yielded significantly shorter times gave results as good as those obtained with the original procedure. The method for electrophoresis used by Gangolli *et al.* (1973) involved the use of cellulose acetate strips. It was found that these strips did not distinguish carrageenans by molecular weight; rather it appeared that in this medium separation occurred almost exclusively by charge-weight ratio. In contrast, the agarose gel was sensitive to the molecular size of the carrageenans, suggesting that molecular sieving occurred. The larger sample volume that could be applied rendered the agarose-gel method somewhat more sensitive than that using cellulose acetate strips.

One drawback of electrophoresis on agarose was the presence of unidentified blue-staining material derived from the biological samples. This interfering material was not eliminated by prior treatment with hyaluronidase or chondroitin sulphatase. When the method used by Beattie *et al.* (1970) for urine analysis was applied to the carrageenans isolated from liver, the tissue blank was found not to interfere. A number of direct standard curves were constructed with different carrageenans (nos 3, 4, 8 and 10–17, Table 1) using the Beattie method, to determine whether or not differences in apparent molecular weight or chain type would cause significant differences in the degree of metachromasia in toluidine blue O. The curves were constructed for concentrations of 0–4·5 μg carrageenan/ml, linear regression analysis was carried out, and the resulting slopes and intercepts were tested for differences. Within a single type of carrageenan, the slopes and intercepts were almost identical, with the exception of the ι-carrageenan of lowest M_n (no. 4) which had a slope significantly different from that of the other iota fractions. The ι- and λ-carrageenans did not differ from each other, a result which might have been expected since the λ-carrageenans had originally been converted, at least partially, to θ-carrageenans. Less metachromasia per unit weight was obtained from the κ-carrageenans than from the

Table 2. *Carrageenan in the livers of rats given ι-carrageenans of various molecular weights*

Rat no.*	Sex	Carrageenan fraction no.†	M_n‡	Metachromasia§
469	M	5	8700	+
567	F	5	8700	+
568	F	5	8700	+
399	M	6	21,000	+
396	M	6	21,000	+
497	F	6	21,000	+ −
511	F	7	39,000	+ −
405	M	7	39,000	+ −
513	F	7	39,000	−
423	M	8	88,000	+ −
424	M	8	88,000	−
526	F	8	88,000	−
438	M	9	107,000	−
538	F	9	107,000	−
539	F	9	107,000	−
451	M	10	145,000	−
453	M	10	145,000	−
522	F	10	145,000	−

* Each carrageenan was administered in aqueous solution by gavage to three rats in doses of 500 mg/kg/day for 9 months.

† See Table 1.

‡ Number average molecular weight.

§ Observed on agarose gel after electrophoresis and staining with toluidine blue O: +, present: −, absent; + −, presence questionable.

others, except for ι-carrageenan no. 4, which gave a response similar to κ-carrageenans. The mixed κ,λ-carrageenans (nos 3 and 17) produced metachromatic changes intermediate between those of pure κ- and λ-carrageenans.

From the above results, it was concluded that possible hydrolytic degradation of carrageenans administered to the animals would not introduce significant errors in the quantitative analysis, unless such degradation progressed to species of very low molecular weight. With method A, overall recoveries of 60–90% were obtained and with method B, recoveries of about 95% resulted.

Analysis of liver specimens

Evidence for the presence of carrageenan in the livers of rats was obtained only in those animals given ι-carrageenans with M_n 88,000 or less for 9 months (Table 2). Even so, the amount of metachromasia visible against the blue background on the stained gels after electrophoresis was small. Samples of livers from rats given carrageenans of M_n 21,000 or M_n 8700 displayed degrees of metachromasia in inverse proportion to their M_n values. No evidence for the presence of carrageenan in liver was found after qualitative examination of samples from rats given ι-carrageenans of M_n 107,000 or 145,000 or after qualitative and quantitative examination of liver samples from rats fed κ,λ-carrageenans of M_n 186,000, 200,000 or 214,000. In comparison with rats, rather more carrageenan was found in the livers of guinea-pigs given ι-, κ- or λ-carrageenans (Table 3), whether the substances were fed in the diet (experiment 1) or given in the drinking-water (experiments 2 and 3). As with the rats, more carrageenan was found in the livers of guinea-pigs receiving carrageenans of low than of high M_n. Animal-to-animal variation was high in all of these studies, as the standard errors indicate (Table 3).

Qualitative analysis by gel electrophoresis indicated that the mobilities of the carrageenans extracted from guinea-pig livers were almost always greater than those of the carrageenan fractions that had been fed (Table 4). Only with carrageenans of very low M_n were the mobilities of administered and recovered carrageenans the same. Comparison of the relative mobilities of ι-carrageenans recovered from guinea-pig livers, regardless of M_n, showed that a remarkable uniformity existed in their electrophoretic behaviour (Table 5). Both the apparent distribution of the carrageenans (i.e. the size, shape and density of their metachromatic stain) and their mobilities were similar to the corresponding properties of the M_n 8700 standard. Thus analysis by gel electrophoresis suggested that ι-carrageenans of M_n about 10,000 were taken up and stored in the livers of guinea-pigs, regardless of the M_n of the ι-carrageenan fed.

In another approach to understanding the effect of molecular weight on uptake, the M_n of the ι-carrageenans was compared to the results of quantitative analysis of liver samples by comparing the distribution of molecular weights in the preparations having various M_n values with the respective amounts of carrageenan found in the liver of guinea-pigs. For each of the ι-carrageenans given to guinea-pigs (Table 3, experiments 1 and 2, method B) linear regression analyses were carried out, relating the amount of carrageenan found in the liver to the fractional amount of that carrageenan lying below some arbitrary M_n threshold. Regressions were run at each of the thresholds listed in Table 6, for both dietary carrageenans and for the carrageenans given in the drinking-water. The liver content of carrageenan was predicted at each point on each regression and a sum of squares representing the difference between observed and predicted values was calculated for each regression. For both experiments, the regression line 'best' fitting the observed data set was chosen, on the basis of the following criteria: (1) the highest correlation coefficient, (2) the lowest sum of squares and (3) an apparently random distribution of observed values on either side of the regression line. The results of these analyses are shown in Fig. 1. The molecular threshold giving the 'best' regression line for the data from Table 3, experiment 1, was 85,000, the correlation coefficient (r) was 0·98 and the regression line was calculated as $y = 743{\cdot}9(x) - 16{\cdot}7$. For the data from Table 3, experiment 2, the molecular threshold was 50,000, r was 0·98 and the 'best' regression line was $y = 592{\cdot}0(x) + 224{\cdot}2$. The regressions relating either M_n or $[\eta]$ values for each ι-carrageenan administered in experiments 1 and 2 (Table 3) to the amount of carrageenan found in the liver were not as good as the corresponding 'best' regression determined above. A similar analysis was not possible for the κ- and λ-carrageenans, because too few individual preparations with different M_n values were available.

Table 3. *Carrageenan in the livers of guinea-pigs given ι-, κ- and λ-carrageenans of various molecular weights*

Experiment no.*	Type of carrageenan	$[\eta]$† (dl/g)	M_n‡	Carrageenan in liver (μg/g)§ Method A	Method B
1	ι	0·285	8700	387 ± 166 (5)	735 ± 285 (2)
		0·685	21,000	445 ± 116 (5)	612 ± 267 (4)
		1·620	39,000	536 ± 180 (5)	506 ± 180 (4)
		4·190	88,000	112 ± 22 (5)	154 ± 43 (5)
		5·340	107,000	+ ‖ (5)	121 ± 59 (3)
		7·510	145,000	+ ‖ (5)	75 ± 9·4 (3)
2	ι	0·285	8700	—	781 ± 265 (5)
		0·685	21,000	—	676 ± 113 (4)
		1·620	39,000	—	386 ± 140 (5)
		4·190	88,000	—	328 ± 193 (3)
		5·340	107,000	—	291 ± 164 (4)
		7·510	145,000	—	212 ± 73 (5)
3	κ	0·177	8500	460 ± 146 (5)	—
		1·451	51,500	348 ± 32 (5)	409 ± 133 (5)
		11·950	314,000	0¶ (4)	0¶ (2)
	λ	0·503	20,800	500 ± 128 (5)	307 ± 107 (5)
		2·243	74,800	510 ± 204 (4)	594 ± 308 (3)
		10·250	275,000	0¶ (3)	0¶ (2)

* In experiment 1, groups of guinea-pigs received one or other of the ι-carrageenans (2% in the diet, 0·5–1 g/kg/day) for 7–10 wk. In experiments 2 and 3, groups of guinea-pigs received one or other of the carrageenans as a 1% solution in drinking-water for 2–3 wk (1–2 g/kg/day).

† $[\eta]$ = intrinsic viscosity (data supplied by Marine Colloids, Inc.).

‡ M_n = number average molecular weight (data supplied by Marine Colloids, Inc.).

§ In method A, quantitation was by densitometry on strips cut from agarose-gel electropherograms. In method B, quantitation was by the method of Beattie, *et al.* (1970). Values presented represent the mean ± SEM for the numbers of determinations indicated in parentheses.

‖ + indicates that metachromatic material was observed in treated but not in control samples, but that the amount present was too small to be quantitated.

¶ 0 indicates that no metachromasia was seen and that all values obtained by quantitative analysis were less than the detection limit of the method (5–10 μg/g liver).

Faecal analysis

No balance studies were attempted, but faecal samples were examined qualitatively, by gel electrophoresis. Since the concentrations of carrageenans present in the faeces were 2–3 orders of magnitude greater than those found in the liver, which on cursory examination of other tissues appeared to contain most of the carrageenan in the body, the faeces must have contained most of the administered dose. With the exception of one anomalous result (carrageenan no. 1, Table 1), it appeared that changes occurred in the administered carrageenans of highest molecular weight (Table 7). Because of run-to-run variation in gel electrophoresis, it proved impractical to estimate the apparent decrease in molecular size, except to suggest that all carrageenans present in faeces had been reduced to M_n values of 100,000 or less (Table 8).

Urine analysis

It appears that excretion of carrageenan in the urine is limited to the smallest of molecular sizes, probably M_n 10,000 or less (Table 9). This conclusion is supported by comparison of the results obtained after giving two monkeys a single oral dose of low-molecular-weight carrageenan (no. 18 (C16), Table 1) with those obtained in monkeys given daily doses of a high-molecular-weight carrageenan (no. 17, Table 1) for 15 months. In the former case, an average of 138 μg carrageenan/ml was excreted on day 1 and 8 μg/ml on day 2, while in the latter case 12 μg/ml was found on each of two consecutive days. The last two values are approximately twice the blank value in the urine of control monkeys and are about at the limit of detectability. Confirmation of the presence of carrageenan by isolation was not attempted.

DISCUSSION

Taken all together, the results of these studies demonstrate that, in the species used, the amounts of carrageenan present in the liver are related to the average molecular weight of the carrageenan fed. The results obtained with guinea-pigs indicate that the amount of carrageenan in the liver may be a linear function of that fraction of an orally-administered carrageenan that is below some upper limit of molecular weight. A 'best' value of that upper limit can be chosen by a process of discrimination based on the statistical attributes of sets of linear regressions for different values of the upper limit. The use of this

Table 4. *The electrophoretic mobility of carrageenans extracted from the livers of guinea-pigs, as compared to that of the carrageenans fed*

Experiment no.†	Type of carrageenan	M_n	Mobility of liver carrageenan/ mobility of carrageenan fed‡ Run 1	Run 2
1	ι	8700	—	0·99
		21,000	1·1	1·12**
		39,000	—	1·37**
		88,000	1·6	1·43***
		107,000	1·8	1·55**
		145,000	1·9	—
2	ι	5000	0·97	
		8700	0·97	
		21,000	1·0	
		39,000	1·17**	
		88,000	1·33**	
		107,000	1·50***	
		145,000	1·65***	
3	κ	8500	1·05	
		51,500	1·04	
		314,000	—	
	λ	20,800	1·05	
		74,800	1·5	
		275,000	—	

† See Table 3 for dosing procedures.

‡ Comparisons were made only of samples run on the same agarose-gel plate (see Experimental).

Insufficient measurements were available for statistical analyses in Run 1, experiments 1 and 3. Elsewhere values marked with asterisks indicate a significant difference between the mobility of the carrageenan fed and that of the carrageenan in the liver: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Table 5. *Relative electrophoretic mobilities of carrageenans recovered from guinea-pig liver**

M_n† of standard sample	Average relative mobility‡ Standard sample Exp. 1	Standard sample Exp. 2	Recovered from liver Exp. 1	Recovered from liver Exp. 2
5000	—	0·82	—	0·79
8700	0·83	0·76	0·83	0·74
21,000	0·73	0·69	0·83	0·69
39,000	0·58	0·68	0·80	0·78
88,000	0·62	0·58	0·83	0·77
107,000	0·45	0·46	0·69	0·70
145,000	—	0·46	—	0·76
Mean	—	—	0·80	0·75

* Guinea-pigs were given ι-carrageenans of different number average molecular weights in the diet or in the drinking-water (see experiments 1 and 2, Tables 3 and 4).

† Number average molecular weight.

‡ Relative mobility as compared to red component of Gelman RBY dye.

Table 6. *Number average molecular weight distribution of undegraded and degraded ι-carrageenans**

M_n	Fraction of the preparation with molecular weight ($\times 10^{-3}$) less than 20	30	40	45	50	55	60	70	80	85	90	100
8700	0·665	0·845	0·940	0·960	0·970	0·980	0·985	0·990	0·995	0·996	0·997	0·998
21,000	0·255	0·420	0·565	0·625	0·680	0·730	0·765	0·830	0·880	0·900	0·925	0·945
39,000	0·090	0·185	0·280	0·325	0·370	0·410	0·450	0·540	0·605	0·635	0·665	0·720
88,000	0·015	0·040	0·070	0·090	0·110	0·130	0·150	0·190	0·240	0·260	0·280	0·320
107,000	0·010	0·030	0·050	0·065	0·075	0·090	0·105	0·140	0·175	0·190	0·210	0·245
145,000	0·002	0·010	0·025	0·035	0·040	0·070	0·060	0·080	0·105	0·115	0·130	0·150

* Prepared from *E. spinosum* and characterized by Marine Colloids, Inc.

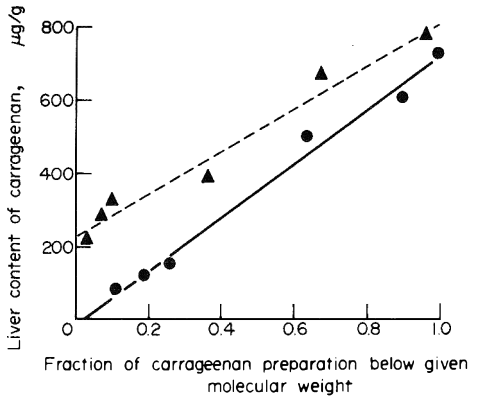

Fig. 1. The effect of the molecular weight of carrageenan on its uptake and retention in the liver. Linear regression analyses were carried out relating the amount of carrageenan found in the livers of guinea-pigs given one of six polydisperse *ι*-carrageenan preparations of different mean molecular weight (M_n) to the fractional amount of each preparation lying below some arbitrary upper limit of M_n. This analysis was done for upper limits of M_n from 10,000 to 100,000 and the best regression was chosen for carrageenan given in the diet (●——●, upper limit 85,000) and in the drinking water (▲- - -▲, upper limit 50,000).

approach provided estimates of the maximum M_n of carrageenan molecules held in the liver of the guinea-pig. With the possible exception of some regional lymph nodes, the liver contains by far the highest concentrations of carrageenan of any tissue (unpublished observations in this laboratory; Grasso *et al.* 1975) and relatively small amounts of carrageenan are excreted (Table 9; Anderson & Soman, 1966; Beattie *et al.* 1970). Therefore characteristics of carrageenans present in the liver should be representative of those absorbed from the gut. When examined directly, the molecular-weight distributions of the carrageenans in guinea-pig livers differed considerably from those of the carrageenans fed, for administered carrageenans having high M_n values. In fact, the M_n distribution of liver carrageenans tends to be quite low, with values of about 10,000. Investigations in this laboratory have failed to produce evidence for the hydrolysis of carrageenans by whole-liver homogenate or by its lysosomal fraction (unpublished results).

When degraded *ι*-carrageenans were administered in the diet, relating the analysis of liver content to molecular-weight threshold values seemed reasonable, intuitively, because the slope-*y* intercept of the regression for the 'best' line almost passed through the origin. The implication was that, in carrageenans containing no molecules of M_n less than that of the threshold value (85,000), no carrageenan would be found in the liver. However, when those same carrageenans were administered in the drinking-water, none of the regression lines passed near the origin. Here the implication was that some carrageenan would be absorbed, whether or not any molecular species below the threshold value (50,000) were present. One might suspect that the behaviour of carrageenan, *vis à vis* absorption and ulceration, is likely to be influenced considerably by the medium in which it is administered, a suggestion in keeping with the complex structure of the molecule and with its propensity to bind to protein. Even the counter-ion composition may have an effect in this regard. The likelihood that these considerations are important is confirmed by the fact that no ulceration or erosion of the gut was noted upon histological evaluation of guinea-pigs or rats given dietary carrageenans,

Table 7. *The electrophoretic mobility of carrageenans extracted from the faeces of various species compared with that of the carrageenan fed*

Species	Type of carrageenan	M_n	Mobility of faecal carrageenan/ mobility of fed carrageenan†
Rat	κ,λ	214,000	0·98 (0·01, 0·03, 8)
	κ,λ	200,000	3·8 (0·12, 0·35, 8)***
	κ,λ	186,000	3·9 (0·05, 0·16, 10)***
Guinea-pig	ι	5000	1·09 (0·02, 0·03, 3)
	ι	8700	1·01 (0·01, 0·01, 9)
	ι	21,000	1·01 (0·01, 0·01, 6)
	ι	39,000	1·11 (0·03, 0·10, 9)
	ι	88,000	1·20 (0·05, 0·15, 9)
	ι	107,000	1·44 (0·10, 0·26, 8)
	ι	145,000	1·36 (0·03, 0·09, 8)***
	κ	8500	1·0 (0·01, 0·01, 5)
	κ	51,500	1·6 (0·02, 0·04, 4)
	κ	314,000	4·4 (0·26, 0·58, 5)***
	λ	20,800	0·99 (0·003, 0·007, 4)
	λ	74,800	1·08 (0·03, 0·05, 3)
	λ	275,000	6·0 (0·39, 0·67, 3)***
Monkey	κ,λ	185,000	6·2 (0·18, 0·37, 7)***

† Ratios of mobility of faecal carrageenan/mobility of fed carrageenan are means, with SEM, SD and the numbers of determinations in parentheses. Comparisons were made only of samples run on the same agarose-gel plate (see Experimental). Values are marked with asterisks where ***$P < 0.001$ for the ratio being different from unity.

Table 8. *Relative electrophoretic mobilities of carrageenans recovered from faecal samples of various species*

Type of carrageenan	Species*	M_n† of standard sample	Average mobilities‡	
			Standard sample	Sample recovered from faeces
κ,λ	Monkey	185,000	0·09	0·67
	Rat	186,000	0·13	0·52
		200,000	0·12	0·45
		214,000	0·51	0·49
κ	Guinea-pig	8500	0·79	0·79
		51,500	0·72	0·77
		314,000	0·10	0·46
κ	Guinea-pig	20,800	0·72	0·71
		74,800	0·61	0·66
		275,000	0·09	0·57
ι	Guinea-pig	5000	0·82	0·89
		8700	0·76	0·78
		21,000	0·66	0·68
		39,000	0·54	0·60
		88,000	0·40	0·48
		107,000	0·28	0·46
		145,000	0·37	0·51

* Rats, guinea-pigs and monkeys were given carrageenans in the diet or drinking-water or by oral gavage, respectively.
† Number average molecular weight.
‡ Relative mobility compared to the red component of Gelman RBY dye.

whereas extensive damage occurred when carrageenans of lower molecular weight were administered in the drinking-water (R. Abraham, personal communication 1973). One must suspect that absorption of carrageenans occurs in the absence of ulceration and possibly, therefore, prior to it. It seems unlikely that the difference in the daily rate of consumption, which was approximately doubled in the drinking-water experiment compared with the diet experiment, was sufficient to account for the marked differences observed in the biological response.

The data presented here confirm the observations of others (Grasso *et al.* 1975; Sharratt *et al.* 1970) that less carrageenan is absorbed by the rat than by the guinea-pig. The resistance of the rat to caecal or colonic ulceration, as compared to the guinea-pig and monkey, is consonant with the above observation and lends support to the hypothesis that the presence of carrageenan in the lamina propria of the caecum and colon is a necessary, though perhaps not sufficient, condition for the production of ulcers.

This report is the first to demonstrate that high-molecular-weight carrageenans are degraded as a result of their passage through the intestinal tract. Where the degradation occurs, whether or not it is inducible and what influence it might have on ulcerogenic potential in different species are questions that need to be explored.

Table 9. *Excretion of carrageenan in the urine of guinea-pigs given ι-carrageenans of various molecular weights in the drinking-water*

M_n*	No. of specimens	Average carrageenan in bladder urine† (μg/total vol.)
5000	2	230
8700	4	320
21,000	2	80
39,000	5	0‡
88,000	3	0‡
107,000	4	0‡
145,000	2	0‡

* Number average molecular weight; data supplied by Marine Colloids, Inc.
† Total carrageenan present in bladder urine at autopsy.
‡ No detectable carrageenan (limit of detection *c.* 15 μg in total urine).

Conclusions

Regardless of molecular weight or of chain-type, the relative amount of carrageenan absorbed and retained in the liver is species-dependent. The amount of carrageenan absorbed and retained in the liver is dependent upon the numerical mean weight (M_n) and the degree of polydispersity of each preparation.

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METHODS FOR LIMITING THE CONTENT OF DIMETHYLNITROSAMINE IN CHINESE MARINE SALT FISH

Y. Y. FONG and W. C. CHAN

Departments of Biochemistry and Pathology, University of Hong Kong, Hong Kong

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Abstract—Dimethylnitrosamine at levels ranging from 0·01 to 0·1 ppm has been confirmed in Chinese marine salt fish by gas chromatography–mass spectrometry. Attempts were made, therefore, to inhibit the formation of nitrosamines in salt fish. Benzoic acid was found to be more effective in reducing the rate of nitrosamine formation than the tetracyclines, which actually increased its production. Salting with chemically pure sodium chloride produced less dimethylnitrosamine than that using crude salt, which commonly contained nitrate. Fresh-water fish were shown to contain a lower level of nitrosatable amines than marine fish and therefore to present a less favourable environment for the formation of nitrosamines in general.

INTRODUCTION

During the past decade, many foods used for human consumption have been reported to contain carcinogenic nitrosamines. Most of the investigations of such foods have been undertaken because a high incidence of tumours in a region has suggested that a particular local practice of food processing might lead to nitrosamine formation. Notable examples of such localized distribution of tumours are high incidences of liver cancer in parts of East Africa and of oesophageal cancer in the Transkei area of South Africa. Du Plessis, Nunn & Roach (1969) identified dimethylnitrosamine in the fruit of the plant *Solanum incanum*, a common additive to foods consumed by the Transkeian Bantu.

In Hong Kong, there is a high incidence of both hepatocellular carcinoma and nasopharyngeal carcinoma among Chinese and it was suspected that this might be related to the dietary customs of the local people. Cantonese salt fish, a common and favourite item of food among Chinese, was studied because the particular way in which it is prepared suggested that nitrosamines might be formed. In the fish itself there are rich sources of secondary and tertiary amines (Rea & Shewan, 1949; Varela & Wojciech, 1956), while the crude sea salt used to pickle the fish contains nitrate and possibly nitrite (Fong & Chan, 1973a). The pickling and drying is done in the open, so the fish is liable to contamination by bacteria. All these factors suggest the possibility that nitrosamines might be formed in the salt fish.

Using the technique of gas chromatography–mass spectrometry, the presence of dimethylnitrosamine (at levels in the 0·01–0·1 ppm range) was confirmed in nine samples of local marine salt fish (Fong & Chan, 1973a). It was also demonstrated that nitrate-reducing staphylococci played an important role in the formation of dimethylnitrosamine in salt fish (Fong & Chan, 1973b). The present communication reports ways in which the formation of dimethylnitrosamine in salt fish can be limited or prevented.

EXPERIMENTAL

Materials. Several species of fresh-water fish and marine fish were used in the experiments described here. The fresh-water fish were mud carp (*Cirrhina molitorella*), grass carp (*Ctenopharyngodon idellus*), grey mullet (*Mugil cephalus*) and big head (*Aristichthys nobilis*); the marine fish were red seabeam (*Argyrops spinifers*), golden thread (*Nemipterus virgatus*), mackerel (*Scomberomorus commersoni*) and yellow croaker (*Pseudosciaena crocea*).

Procedures

Determination of nitrosatable amine in marine and fresh-water fish. This analysis was performed on three marine and three fresh-water species (Table 1). In each case, two different 100-g samples of fish were used. The determination of nitrosatable amines in fish was based on the drastic nitrosation of amines with a high level of sodium nitrite (0·145 M) at pH 3 (Walters, Newton, Parke & Walker, 1974) and the

Table 1. *Levels of nitrosatable amines in marine and fresh-water fish*

Fish samples	Total nitrosation detected* (μmol *N*-nitroso derivative/kg fish)
Marine	
Red sunbeam	126; 443
Golden thread	119; 85
Mackerel	45; 52
Fresh-water	
Mud carp	4; 7
Grass carp	24; 15
Grey mullet	34; 11

*Volatile plus non-volatile CH_2Cl_2-extractable fraction; calculated on the basis of one nitrosatable group/mol amine, determined colorimetrically after drastic nitrosation with nitrite at pH 3 and reaction with HBr in glacial acetic acid.

Table 2. *Level of dimethylnitrosamine in marine and fresh-water fish following nitrosation*

Fish samples	Nitrosation detected (ppm dimethylnitrosamine*)	
	Fish-residue fraction	Fish-supernatant fraction
Marine		
Red sunbeam	0·060; 0·010	38·00; 45·00
Golden thread	0·750; 0·140	6·00; 5·50
Mackerel	0·006; 0·008	1·70; 2·40
Fresh-water		
Mud carp	<0·001; <0·001	0·400; 0·090
Grass carp	<0·001; <0·001	0·200; 0·080
Grey mullet	0·008; 0·001	0·020; 0·200

*Determined by gas chromatography, following drastic nitrosation by $NaNO_2$ at pH 3, and confirmed by gas chromatography–mass spectrometry.

measurement of the extent of nitrosation by the colorimetric method of Eisenbrand & Preussmann (1970), in which hydrogen bromide in glacial acetic acid was used to cleave *N*-nitroso compounds to produce nitrite in quantitative yield. The liberated NO^+ was captured by diazotation of sulphanilic acid and the resulting diazonium ion was coupled with *N*-[naphthyl-1]-ethylene diammonium dichloride in 30% glacial acetic acid. The extent to which nitrosation occurred was also estimated from the level of dimethylnitrosamine by using gas–liquid chromatography.

Preparation of salt fish in the laboratory. Samples of salt fish were prepared either with crude salt containing 40 ppm nitrate, with pure NaCl, with NaCl and $NaNO_2$ or with NaCl and $NaNO_3$. Fish of two marine and two fresh-water species were obtained from local retailers. Fish of each species were weighed and salted with the pickling agents shown above. The amount of salt used was 20% (w/w) of the fish and the amount of nitrate or nitrite 200 ppm. The fish were pickled for 1–2 days with layers of salt packed between them. They were then dried in the open air under the sun for a few days, depending on the weather. Just prior to the drying, solid salt was forced into the mouth and between the gills. The fish were then put away and stored for 4–5 months (the usual time between pickling and selling) before being analysed for the presence of dimethylnitrosamine according to the method of Sen, Smith, Schwinghamer & Howsam (1970).

Incorporation of antimicrobial preservatives in the salting process. A batch of about 9 kg fresh golden thread was purchased from the local market. These fish were divided into nine approximately equal lots and salted, respectively, with crude salt, with crude salt and chlortetracycline, with crude salt and tetracycline, with crude salt and oxytetracycline, with crude salt and benzoic acid, with NaCl, with NaCl and chlortetracycline, with NaCl and oxytetracycline, or with NaCl and benzoic acid. The amount of salt used was 20% (w/w) of fish and the antimicrobial preservatives were incorporated at a level of 20 ppm in the salt (Vaughn & Stewart, 1960). The usual method of preparation (as described above) was followed. Dried salt fish was then analysed for the presence of dimethylnitrosamine by the method of Sen *et al.* (1970).

RESULTS

It can be seen from Table 1 that the levels of nitrosatable amines were higher in the marine fish than in the fresh-water fish. Also, the level of dimethylnitrosamine in marine fish was correspondingly higher after nitrosation with 0·145 M-$NaNO_2$ at pH 3 (Table 2).

The amounts of dimethylnitrosamine formed in marine and fresh-water fish preserved with crude salt, with pure NaCl, with NaCl and $NaNO_2$ or with NaCl and $NaNO_3$ are shown in Table 3. It can be

Table 3. *Level of dimethylnitrosamine in marine and fresh-water fish prepared by various salting procedures*

Fish	Dry weight (g)	Levels of dimethylnitrosamine* (ppm) in fish preserved with			
		Crude salt	Pure NaCl	NaCl + $NaNO_2$	NaCl + $NaNO_3$
Marine					
Yellow croaker	500 ± 10	0·040	0·006	0·200	0·130
Golden thread	200 ± 10	0·025	0·007	0·065	0·060
Fresh-water					
Mud carp	500 ± 10	0·020	0·005	0·057	0·050
Big head	200 ± 10	0·019	ND	0·030	0·037

ND = Not detected

*Identity confirmed by gas chromatography–mass spectrometry.

Table 4. *Level of dimethylnitrosamine in golden thread preserved with crude salt or NaCl in conjunction with antimicrobial preservatives*

Preservative	Level of dimethyl nitrosamine* (ppm)
Crude salt	0·047
Crude salt + chlortetracycline	0·110
+ tetracycline	0·060
+ oxytetracycline	0·052
+ benzoic acid	0·012
NaCl	0·008
NaCl + chlortetracycline	0·065
+ oxytetracycline	0·080
+ benzoic acid	ND

ND = Not detected
*Identity confirmed by gas chromatography–mass spectrometry.

seen that when either $NaNO_2$ or $NaNO_3$ was added at a level of 200 ppm, the amount of dimethylnitrosamine formed in both marine and fresh-water fish was higher than when these compounds were not included. In all cases, the amount of dimethylnitrosamine formed in fresh-water fish was less than that in marine fish.

The amount of dimethylnitrosamine formed in golden thread salted with added antimicrobial preservatives is shown in Table 4. For preventing the formation of dimethylnitrosamine, benzoic acid proved a better preservative than the tetracyclines. The use of various tetracyclines increased the yield of dimethylnitrosamine in salt fish.

DISCUSSION

It is extremely difficult to assess in terms of human epidemiology the significance of the presence of dimethylnitrosamine in salt fish. The concentrations confirmed in salt fish (0·01–0·1 ppm) are far below those known to have a carcinogenic action in experimental animals. Terracini, Magee & Barnes (1967) reported that when rats were fed a diet containing as little as 2 ppm dimethylnitrosamine for a lifetime, tumours developed in the liver, mouth, mammary gland, kidney and lung. However, the effect of small doses administered for long periods is by no means well studied or understood, and human conditions are difficult to reproduce in other species. It is safer, therefore, to adopt the view that no dose of a carcinogen, however small, can be regarded as completely safe.

From the experimental results in this report, it seems likely that the possible levels of dimethylnitrosamine in salt fish could be reduced in three ways, namely by limiting the concentrations of the carcinogen precursors (amine and nitrate or nitrite), by adding antimicrobial preservatives with the salt and by improving the techniques of drying and storing salt fish and other aspects of its preparation. Since nitrosamines can be formed easily from precursors at acid or neutral pH in the presence of bacteria (Collins-Thompson, Sen, Aris & Schwinghamer, 1972; Hawksworth & Hill, 1971; Sander, 1968) the availability of precursors is a determining factor in the formation of nitrosamines.

It can be seen from Table 1 that marine fish are richer in nitrosatable amines than are fresh-water fish. The degree of nitrosation detected (expressed as μmols *N*-nitroso derivatives/kg fish, on the basis of one nitrosatable group/mol amine) varied from 45 to 443 and from 4 to 34 for three species of marine and fresh-water fish, respectively. The level of dimethylnitrosamine in marine fish (1·7–45 ppm) was also much higher than that (0·02–0·4 ppm) in fresh-water fish (Table 2). Although allowance should be made for factors such as the age, species and condition of the fish studied, these findings indicate that marine fish present a richer source of nitrosatable amines than fresh-water fish.

The amount of nitrate or nitrite present in the pickling salt is crucial. In laboratory-prepared marine salt fish, the amount of dimethylnitrosamine formed with NaCl and 200 ppm $NaNO_2$ or $NaNO_3$ as the pickling agent was 3–5 times higher than when crude salt containing 40 ppm nitrate was used (Table 3). It should be noted that only trace amounts of dimethylnitrosamine (0·006 ppm) were formed when chemically pure NaCl was used. Moreover, it could be seen that the levels of dimethylnitrosamine in fresh-water fish were always lower than those in marine fish prepared in an identical manner, although levels in golden thread (marine) and mud carp (fresh-water) were relatively close. This may have been due to the fact that this particular sample of golden thread was low in nitrosatable amine content because it was stale when pickled.

It appears, therefore, that if the dimethylnitrosamine content of salt fish is to be reduced, chemically pure NaCl should be used as the pickling agent instead of nitrate-containing crude sea salt. Similarly, fresh-water fish with lower concentrations of nitrosatable amines are preferable to marine fish.

Since bacteria isolated from salt fish have been demonstrated to enhance significantly the yield of dimethylnitrosamine in salt-fish broth (Fong & Chan, 1973b), it would seem logical to add antimicrobial preservatives in the salt as a means of reducing nitrosamine formation. In this connexion, the potentialities of various preservatives have been explored in the present study. From Table 4, it can be seen that benzoic acid is a better preservative than the tetracyclines as far as restricting the formation of dimethylnitrosamine is concerned. In fact, the use of various tetracyclines increased the production of dimethylnitrosamine in salt fish. On closer examination, this is not surprising, as these antibiotics are tertiary amines which are capable of forming nitrosamines with nitrite under suitable conditions (Lijinksy, Conrad & Van de Bogart, 1972). In the present work, no nitrosamine was detected when NaCl was used in combination with benzoic acid. These findings indicate the difficulty of finding a suitable antimicrobial food preservative; it appears that benzoic acid is better than the antibiotics studied.

Bacterial contamination can be further reduced by improving techniques of salt-fish processing. Though sun-drying is cheap, it is not a process suitable for Hong Kong, where the relative humidity is high throughout the year and where it is impossible to

exclude contamination by maggots and flies during sun-drying. As a result, mechanical drying is recommended. In the construction of the drying cabinet itself and in the working conditions aimed at, Hong Kong could be guided by the pioneer work of Linton & Wood (1945) of the Atlantic Station of the Fisheries Research Board of Canada.

Bacterial contamination would be further reduced if the fish were cleaned and gutted before being pickled, and if proper storage devices such as canning or sealing in bottles were introduced.

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REPRODUCTIVE DYSFUNCTION IN RHESUS MONKEYS EXPOSED TO LOW LEVELS OF POLYCHLORINATED BIPHENYLS (AROCLOR 1248)

D. A. BARSOTTI, R. J. MARLAR and J. R. ALLEN

Department of Pathology, University of Wisconsin Medical School, Department of Food Microbiology and Toxicology, and Regional Primate Research Center, University of Wisconsin, Madison, Wisconsin 53706, USA

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Abstract—Eighteen female and four male adult rhesus monkeys were fed the polychlorinated biphenyl (PCB) Aroclor 1248 at levels of either 2·5 or 5·0 ppm in the diet. These levels are equal to, and 50% of, the concentration allowed in certain foods destined for human consumption. After consuming these diets for 2 months, some of the females developed acne, alopecia, erythema and swelling of the eyelids, and by 6 months all females exhibited these changes to some degree. Modifications in serum lipids developed gradually, with a trend towards hypocholesterolaemia, hypolipidaemia and decreased serum triglycerides. In addition there was a shift in the plasma albumin/globulin ratio and an increase in serum glutamic-pyruvic transaminase activity. Analysis of subcutaneous fat showed an accumulation of the PCB isomers in the adipose tissue. The concentrations in this tissue reached a plateau, after which only slight variations were observed. Within 4 months, menstrual cycles were altered; menostaxis and menorrhagia occurred frequently and at times amenorrhoea was apparent. The ability of the animals to maintain pregnancy was impaired, as indicated by frequent resorptions and abortions. When infants were born they were small, and the transplacental movement of PCBs was evident from analyses of skin biopsies of neonates and of autopsy tissue from one stillborn. Moreover, additional accumulation of PCBs occurred in infants during breast feeding. All males fed 5·0 ppm PCB exhibited only slight periorbital oedema and erythema after 14 months on the diet and showed no alterations in their breeding capacities. The data presented indicate that long-term, low-level exposure of female non-human primates to PCBs can affect many important biological parameters.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are some of the more widespread and injurious environmental contaminants. These industrially produced compounds have been used extensively, because of their heat resistance, dielectric properties and extreme stability. The properties that ensure their usefulness to industry also make them very resistant to biodegradation. The main routes of environmental contamination by these compounds include open burning, vaporization, accidental spillage and waste disposal in sewage. In addition to acne, which is a fairly constant complication in most mammalian species exposed to PCBs, these compounds have caused reproductive failure in mink of the Great Lakes region (Aulerich, Ringer & Iwamoto, 1973; Ringer, Aulerich & Zabik, 1972), in sea lions on the Pacific Coast (DeLong, Gilmartin & Simpson, 1973), in birds in many areas of the world (Risebrough, Rieche, Peakall, Herman & Kirven, 1968) and in man following the industrial contamination of rice oil in Japan (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972). In the Japanese incident, the occurrence of abortions, stillbirths and undersized infants was attributed to the transplacental movement of the PCBs. Subsequent research (Allen, Carstens & Barsotti, 1974a; Curley, Burse, Grim, Jennings & Linder, 1971) has shown that the PCBs do, in fact, pass across the placental barrier and are excreted in the milk of lactating mothers (Miller & Fox, 1973; Musial, Hutzinger, Zitko & Crocker, 1974).

Although the utilization of PCBs has been limited to closed systems, the level of contamination, particularly in bodies of water adjacent to densely populated areas, is not declining and in many instances appears to be increasing. Thus, the PCBs remain a major environmental contaminant of significance to health. In the present report some of the possible ramifications of low-level, long-term exposure to PCBs are presented. Even at the levels currently permitted in foods destined for human consumption, the PCBs are capable of producing widespread harmful effects in female primates.

EXPERIMENTAL

Animals and diets. Thirty female and ten male adult rhesus monkeys were housed in a controlled environment simulating the light, humidity and temperature conditions of the breeding season for these feral animals in India. During the 6 months prior to the administration of the experimental diets, the menstrual cycles, sperm counts and morphological features of the spermatozoa were evaluated in these animals. The monkeys were subsequently placed in three groups, a control group of 12 females and six males given a commercial diet (Ralston-Purina Co., St. Louis, Mo.), a second group of nine females receiving a similar diet to which 2·5 ppm PCB (Aroclor 1248, Monsanto Co. Inc., St. Louis, Mo.) was added, and a third group of nine females and four males receiving a similar diet containing 5·0 ppm PCB. The exper-

imental diets were prepared by adding the appropriate levels of PCB to a finely powdered meal and subsequently pelleting the mixture into 0·5 × 1·25 in. cylindrical pellets. Periodically, samples were analysed by gas–liquid chromatography to confirm the PCB level. The females on the experimental and control diets received 200 g daily, while all males received 300 g of their respective diets daily. All diets were supplemented with fresh fruit and the animals received water *ad lib.* The food left in the feeder was removed and weighed daily for calculation of intake.

Breeding study. After 7 months on the experimental diets, the eight surviving females from the 2.5-ppm group, eight females from the 5-ppm group and the 12 control females were allowed to breed. Ovulation was predicted from observation of menses and previous menstrual cycle length (Valerio, 1969). In general, the experimental females were housed with control males for 5 days during the appropriate time of ovulation, and the experimental males were housed with control females for a similar period. Pregnancy tests on females were performed according to Wilson, Fradkin & Hardman (1972). The findings were confirmed by rectal palpation of the uterus at 40 days of gestation.

Observations and analyses. Throughout the experiment, animals were observed daily for any physical change and were weighed monthly. Serum triglycerides, cholesterol, serum glutamic–pyruvic transaminase (SGPT), albumin/globulin ratios, total lipid and haemograms were determined monthly in all animals (Allen, Norback & Hsu, 1974b). In addition, every 3 months an inguinal subcutaneous fat biopsy taken from selected animals in each group (Allen *et al.* 1974a) was extracted and evaluated for PCBs according to a modification of the procedure originally presented by Curley *et al.* (1972). For chromatographic analysis, fat samples were homogenized in hexane (500 mg/20 ml) and subsequently evaporated under dry nitrogen in a 40°C waterbath. Clean-up of the extracted material prior to analysis was carried out in a disposable pipette microcolumn containing silica gel 60 (0·05–0·2 mm) by elution with a benzene–hexane (1:1, v/v) mixture. Levels of PCBs were estimated in a Hewlett-Packard 7620A chromatograph employing a ^{63}Ni electron-capture detector. A pyrex column packed with Gaschrom Q (80–100 mesh) coated with 2% SE-30 was operated at 170°C with argon-methane (95:5) as the carrier gas and at a flow rate of 40 ml/min.

Table 1. *Changes in the body weight of female monkeys receiving a diet containing 2·5 or 5·0 ppm Aroclor 1248*

Time on diet (months)	Mean body weight (kg)	
	Control group	Experimental group
0	4·50 ± 0·68	5·57 ± 0·55
1	4·89 ± 0·60	5·47 ± 0·58
2	4·86 ± 0·41	5·28 ± 0·55
3	5·00 ± 0·55	5·38 ± 0·60
4	5·00 ± 0·51	5·38 ± 0·60
5	5·01 ± 0·59	4·84 ± 0·48

Values are means ± standard deviation for groups of 12 control or 18 PCB-fed (2.5 or 5 ppm) animals.

RESULTS

During the first 6 months that the females received PCBs in their diets, the intake was relatively constant, with the animals ingesting an average of 90 and 180 mg PCB on the 2·5 and 5·0 ppm diets, respectively. Despite normal food consumption, both experimental groups lost an average of 15·1% of their initial body weight (Table 1). Within 2 months of the start of the experiment and when 30–60 mg PCB had been consumed, individual animals began to show the typical signs of PCB intoxication, including loss of hair, acne of the face and neck, and erythema and swelling of the eyelids. At 6 months all females exhibited these signs to some degree. Skin biopsies revealed affected hair follicles with prominent keratinization. The haematological changes developed more gradually. Haemograms remained relatively normal throughout the period, but the concentrations of serum triglyceride, cholesterol and total lipids decreased progressively (Table 2), although only the decrease in total lipids was statistically significant. There was also a shift in the albumin/globulin ratio and a progressive increase in SGPT activity (Table 2).

The data obtained from the fat biopsies on the female animals (Table 3) showed an accumulation of PCB isomers in the adipose tissue. After a 6-month exposure period, the PCB level in the adipose tissue of the animals receiving 5 ppm attained a plateau, subsequent values varying little with continued consumption of the diet. A similar level was attained in 1 yr by the group given 2·5 ppm PCB.

After 4 months on the experimental diets and consumption of a total of 60–120 mg PCB, menstrual

Table 2. *Modifications in the serum chemistry of female monkeys given a diet containing 2·5 or 5·0 ppm Aroclor 1248*

Time on diet (months)	Serum levels (mg/100 ml)			Albumin/ globulin ratio	SGPT activity (RFU)
	Triglycerides	Cholesterol	Total lipids		
0	75 ± 18	166 ± 28	582 ± 90	1·20 ± 0·19	14 ± 3
2	93 ± 48	152 ± 28	573 ± 98	1·23 ± 0·20	24 ± 7
3	75 ± 24	159 ± 24	581 ± 97	1·30 ± 0·21	21 ± 8
4	51 ± 20	156 ± 26	451 ± 74	1·23 ± 0·16	21 ± 9
8	61 ± 20	156 ± 22	470 ± 83†	1·08 ± 0·19*	31 ± 7**

SGPT = Serum glutamic-pyruvic transaminase RFU = Reitman-Frankel units

Values are means ± standard deviation for a group of 16 animals (eight given 2·5 ppm and eight 5·0 ppm PCB) and asterisks indicate those 8-month values that differed significantly from the value at month 0: *$P < 0·05$; **$P < 0·001$.

Table 3. *Total intake and adipose-tissue levels of PCBs in female monkeys consuming a diet containing 2·5 or 5·0 ppm Aroclor 1248*

Dietary level (ppm) of PCB	No. of animals	Values after administration of PCB diets* for 1 month: Total PCB intake (mg)	1 month: Tissue content of PCBs (μg/g)	6 months: Total PCB intake (mg)	6 months: Tissue content of PCBs (μg/g)
2·5	4†	15·63 ± 2·63	5·06 ± 3·50	85·29 ± 8·07	32·69 ± 15·97
5·0	3	30·95 ± 1·06	16·39 ± 4·49	175·36 ± 4·24	71·30 ± 31·56

*Data have not been included on the PCB levels of fat obtained from the control animals because in all instances the minor peaks recorded in the gas-chromatographic analysis could not be stated to be PCBs. Furthermore the amount they represented was infinitesimally small.

†One animal in this group showed lesions early in the experiment and gas-chromatography of its adispose tissue revealed that the PCB concentrations were closer to those of the animals fed 5·0 ppm despite the lower intake of PCB. This animal was not included in the calculations.

Values are means ± standard deviation for the numbers of animals shown.

cycles were decidedly altered, particularly in the group receiving the 5-ppm diet. There were increases in the duration of menses and in menstrual bleeding. Menses were 5–7 days longer in the experimental group than in the controls. Several of the experimental animals exhibited occasional absence of menstrual bleeding.

Conception rates for the control animals and for those fed 2·5 ppm PCB were essentially the same, but, the conception rate in the animals receiving 5 ppm was decreased. As shown in Table 4, eight animals on the 2·5-ppm diet became pregnant, as determined by the level of chorionic gonadotropin in mouse bioassays, but shortly afterwards three of these animals resorbed their embryos. In these animals, implantation bleeding, which usually occurs 17 days after conception, was replaced by profuse uterine haemorrhage. In the 5·0-ppm group, six of the animals were impregnated. The remaining two animals were bred five times without success. Of the animals that conceived in this group, there were abortions at 46, 67 and 107 days of gestation, one resorption, one stillborn and one uncomplicated birth. Viable infants born to the experimental animals were small (1–2 standard deviations from the normal established by Kerr, Scheffler & Waisman, 1969), but were otherwise normal. These observations are in contrast to those in the control animals, all of which conceived and gave birth to normal infants (Table 4).

One adult female on the 2·5-ppm diet and one on the 5·0-ppm diet died after 173 and 310 days of feeding, respectively. At autopsy, these animals showed the typical signs and tissue alterations of PCB intoxication. The major gross findings at autopsy included generalized alopecia, subcutaneous oedema and acne. Microscopically, in addition to hyperplasia of the follicular epithelium and inflammation of the surrounding tissue, hair follicles were filled with keratin. Throughout the liver there were focal areas of necrosis. Hepatocytes were enlarged and contained numerous small lipid droplets. Large lipid droplets prevailed in the cytoplasm of the Kupffer cells. In addition, both animals developed terminally a severe enteritis from which *Shigella flexneri* type IV was isolated. This organism is known to be a common pathogen of primates, but is generally sensitive to treatment. Even following treatment, the animals in this study experienced shigellosis on numerous occasions, a finding consistent with previous observations that PCB-intoxicated animals appear to be more susceptible to this opportune pathogen.

The distribution of PCBs in the tissues of the two females that died, as well as in those of the dead infant born to a mother on the 5·0-ppm diet, is shown in Table 5. The affinity of PCB for lipid-containing tissue is readily apparent and a transplacental movement of PCBs is indicated by the high concentration in the tissues of the stillborn infant. Furthermore the levels of PCBs in the other infants continued to increase after birth as a result of the high concentrations in the milk of the mothers (275·0 ± 121·5 ng/g milk). The level of PCB in the skin obtained by biopsy on the day of birth from infants born to the mothers on the 2·5-ppm PCB diet

Table 4. *Modification in reproduction in primates exposed to Aroclor 1248 in the diet*

Reproduction parameter	No. recorded in groups fed diets containing PCB levels (ppm) of: 0	2·5	5·0
No. of animals/group...	12	8	8
Total impregnated	12	8	6
Resorptions or abortions	0	3	4
Stillborn	0	0	1
Normal births	12	5	1

Table 5. *PCB content of tissues obtained at autopsy from two adult female monkeys and a stillborn infant*

Tissue	PCB content (μg/g) in tissues from		
	Adults fed dietary levels (ppm) of		Stillborn infant‡
	2·5*	5·0†	
Pancreas	0·16	53·89	63·85
Liver	5·61	24·38	2.47
Adrenal	3·09	40·18	24·85
Kidney	0·60	52·77	2·47
Spleen	1·55	30·15	12·52
Small intestine	0·77	3·22	15·00
Stomach	2·68	47·45	18·96
Brain	1·58	21·41	0·34
Lung	3·20	8·69	99·36
Muscle	0·19	17·66	4·48
Skin	0·98	10·03	0·001

*Aroclor 1248 intake: 75·3 mg over 173 days.
†Aroclor 1248 intake: 284·56 mg over 310 days.
‡Aroclor 1248 intake, by mother: 394·3 mg over 401 days on 5·0-ppm diet

was 2·8 ± 1·4 μg/g tissue. This value had increased to 111·6 ± 29·0 μg/g skin after breast-feeding had continued for 3 months.

One of the male animals given 5·0 ppm PCB exhibited a slight periorbital oedema and erythema of the eyes after 6 months (total PCB intake, 300 mg). Only the consumption of approximately 670 mg PCB over 14 months resulted in the development of slight to moderate periorbital oedema and congestion in all of the exposed males. Levels of PCB continued to increase in the adipose tissue of the males with time. In spite of these rises there was no alteration in their gross breeding capabilities (sperm count or breeding success), control females conceived at the same rate whether bred to experimental or control males.

DISCUSSION

The levels of PCB that were incorporated in the diets of experimental animals were selected to represent concentrations equal to or half as great as those permitted in certain foods destined for human consumption. Previous experiments reported from this laboratory have shown that PCB concentrations as low as 25 ppm in the diet of non-human primates may produce morbidity and mortality after 2 months of exposure (Allen *et al.* 1974a).

Skin changes appeared in animals receiving 2·5 or 5·0 ppm PCB as rapidly as in the animals fed larger doses (Allen *et al.* 1974a; Allen & Norback, 1973). In fact, these skin changes were recorded in some animals after a total of only 30–50 mg PCB had been ingested. These data suggest that only short exposure to PCBs is required to stimulate follicular epithelial hyperplasia, blockage of the sebaceous ducts and keratinization of the hair follicles. The early heavy localization of PCB in the skin of affected animals (Yoshimura & Oshima, 1971) is undoubtedly responsible for such tissue changes.

The values for SGPT, albumin/globulin ratios and total lipids were significantly different from the controls at month 8. Although there were indications much earlier, it required 12 months on the experimental diets before changes in the serum triglyceride and cholesterol concentrations were statistically significant. These observations substantiate the findings of Allen *et al.* (1974a) who recorded similar changes in the serum chemistry of monkeys fed 25 ppm PCB for 2 months.

Male monkeys fed a diet containing 5·0 ppm PCB invariably ate more food and attained higher levels of PCB in the adipose tissue than their female counterparts. However, the female monkeys developed signs of PCB intoxication within 2 months whereas only minor skin alterations developed in males after 1 yr. One explanation for this difference in PCB response between the two sexes may be related to the total reservoir of fat tissue. Since the males were much larger than the females, they had a greater quantity of adipose tissue in which the ingested PCB could be sequestered. Platonow & Karstad (1973) described similar differences in the toxic response of mink to PCB, noting that male mink at all treatment levels were less severely affected, survived longer and produced abundant, viable spermatozoa throughout the experiment. Gish & Chura (1970) observed that heavier quail survived longer than lighter ones when treated with DDT.

One of the most serous results of PCB exposure in the adult female monkey was the effect on reproduction. Even with low dietary levels of PCBs, the menstrual cycles were irregular and there was excessive and prolonged menstrual bleeding. In addition, animals that conceived showed a higher incidence of resorption and early abortion even when compared with the 10% abortions and stillbirths recorded each year in the breeding colony. A likely explanation for these changes is related to an oestrogen-progesterone imbalance in these animals, these hormones being responsible for menstrual flow and the maintenance of pregnancy. Preliminary observations from this laboratory suggest an endocrine disturbance resulting from PCB intoxication in females. When females are fed PCB in their diets they invariably have much higher levels of urinary ketosteroids than control monkeys (Barsotti & Allen, 1975).

Another interesting observation was the reduced size of the infants born at term to PCB-fed monkeys. These findings are in agreement with reports from Japan, where the children born to exposed mothers were reduced in size (Kuratsune *et al.* 1972). Even though the infant monkeys had shorter long bones, smaller head circumference and reduced crown-to-rump lengths, the osseus development as viewed radiographically was similar to that in the control infants (unpublished observations). Histologically, there was also normal development of the tissues in day-old infants born to PCB-fed mothers (Allen *et al.* 1974a).

Recently it has been shown that at least a portion of the PCBs are metabolized through an arene oxide intermediate (Hsu, Van Miller & Allen, 1975). There is a distinct possibility that many of the effects that have been recorded in this experiment may be related to an interaction of these epoxides with cellular macromolecules. These findings also suggest that PCB exposure may have long-range effects in primate species, since epoxides are known to be capable of producing necrogenic, carcinogenic and mutagenic changes in mammalian tissues.

It is unlikely that man will be exposed to levels of 2·5 or 5·0 ppm PCBs on a continuous basis in his diet. However, there may be instances when the intake of PCBs may exceed these concentrations for short periods or when exposure over a prolonged period may give rise to toxic concentrations in the tissues. Whether human exposure to the PCBs is sufficiently great to produce serous effects such as reproductive insufficiency or modifications in the functional capacities of certain cellular macromolecules has not been determined. However, the data presented in this report suggest that present levels of PCB contamination may be sufficient to cause such effects.

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TOLERANCE AND ACCEPTABLE DAILY INTAKE OF CHLORINATED FUMIGANTS IN THE RAT DIET*

E. ALUMOT (OLOMUCKI), E. NACHTOMI, E. MANDEL and P. HOLSTEIN
Agricultural Research Organization, The Volcani Center, Bet Dagan,

A. BONDI
Faculty of Agriculture, Rehovot,

and

M. HERZBERG
Ichilov Hospital, Tel Aviv, Israel

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Abstract—Long-term trials were carried out with rats fed mash fumigated with carbon tetrachloride or ethylene dichloride (1,2-dichloroethane). The difficulty of maintaining a constant residual level of fumigant in the feed was overcome by adopting suitable storage and feeding procedures. The amounts of fumigant actually consumed accounted for 60–70% of the residue initially present in the mash. Triglyceride accumulation in the liver was the most sensitive sign of chronic poisoning with carbon tetrachloride and was apparent after only 6 wk of treatment with a daily dose of about 1% of the LD_{50}. Ethylene dichloride caused only a slight increase in liver fat at very high doses. Carbon tetrachloride and ethylene dichloride were each administered to rats for 2 yr in fumigated mash at levels below the obviously harmful ones. Growth, fertility, reproduction and biochemical tests were used as criteria for establishing the no-effect levels. On the basis of these data, acceptable daily intakes of 10 mg/kg body weight for carbon tetrachloride and 25 mg/kg for ethylene dichloride are proposed. The respective tolerances are 100 and 250 ppm. The proposed tolerances in human food are discussed.

INTRODUCTION

Fumigation of cereals with haloalkanes is a common practice to prevent storage losses due to insect infestation. The most frequently used fumigants in this group, methyl bromide, ethylene dichloride (dichloroethane; EDC), ethylene dibromide (dibromoethane; EDB) and carbon tetrachloride (CCl_4) are highly toxic to living organisms. Despite their volatility, all fumigants except methyl bromide leave unchanged residues, which persist for up to several months after fumigation (Bielorai & Alumot, 1966; Olomucki & Bondi, 1955; Scudamore & Heuser, 1973). Residues of EDB in cereal grain were found to affect egg weight and egg production in laying hens (Bondi, Olomucki & Calderon, 1955) and spermatogenesis in the bull (Amir & Volcani, 1965). Levels as low as 1–2% of the LD_{50} were effective, when fed daily in the fumigated feed for several weeks.

It was clear that the fumigant residues in grain could cause chronic poisoning, differing in effects from acute poisoning and specific for each species and compound. The chronic effects are of special importance in animal nutrition since, in contrast to man, the animals are fed unprocessed grain. Although baking or cooking reduce the residues in human food to low levels, the safety of the repeated consumption of such levels is still to be proved. Data indicating no-effect levels of residual fumigants for man and animals are lacking. The 1968 Joint FAO Working Party/WHO Expert Committee Meeting concluded that the available toxicological evidence was insufficient to enable calculation of an acceptable daily intake for several fumigants, and recommended that long-term feeding trials should be carried out in two mammalian species. One of the reasons for the lack of such research is the difficulty of maintaining constant residue levels in a fumigated food. Kenaga, McCollister, Spencer & Torkelson (1969) observed correctly that other methods of dosing were not representative of normal conditions of consumption. For the study now reported, special storage and feeding procedures, suitable for long-term trials, were established, and rats were fed over a 2-yr period with mash fumigated with EDC or CCl_4.

EXPERIMENTAL

Fumigation of the mash. In preliminary experiments designed to demonstrate the no-effect levels, 3-kg batches of commercial mash were fumigated in special 20-litre fumigation containers (Alumot & Calderon, 1965). The levels tested were 150–520 ppm for CCl_4 and 300–1600 ppm for EDC. For the 2-yr trials, commercial mash was fumigated for 48 hr in hermetically sealed 3-litre containers. The following residue levels were assayed: EDC, 250 ± 30 ppm (low level) and 500 ± 40 ppm (high level); CCl_4, 80 ± 5 ppm (low level) and 200 ± 20 ppm (high level).

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The fumigated mash was allowed to aerate for 5 min, to enable the 'free' fumigant to evaporate from the air spaces between the mash particles. It was then distributed either into polyvinyl bags, coated inside with polyamide to prevent loss of fumigant by sorption on to the polyvinyl, or into glass jars sealed hermetically with a plastics screw cover. The inside of the cover was inlaid with a polyamide sheet. The food was stored for 7–10 days, and checks of the residue concentration showed that about 5% was lost between the beginning and end of the storage period. The residues were determined by the cold-extraction method of Heuser & Scudamore (1969).

Feeding procedure. Following studies designed to determine the feeding procedure that involved minimal loss of fumigant, the animals were fed weighed portions twice daily from 11–12 noon and from 7–9 p.m. The evening portion comprised 80% of the daily intake, since rats are nocturnal animals. About 66% of the feed was consumed during the first hour after distribution, and practically no feed remained after the second hour. During the day the animals had access to feed for 1 hr only and became accustomed to consuming it quickly. Any remaining food was removed after each feed and weighed weekly. Fumigant residues in the mash were tested at the beginning of the distribution, and after 1 and 2 hr in the feed troughs. Since the amount eaten and the residue level were known, the amount of fumigant actually consumed was calculated with fair accuracy. It comprised 60–70% of the residue present initially in the feed.

Preliminary study. Groups of six weanling rats aged 4 wk were fed diet containing 150, 275 or 520 ppm CCl_4 for 6 wk (males) or 5 wk (females) at the end of which the animals were weighed and killed and the livers were analysed for total lipids, triglycerides and phospholipids (the latter in the females only). Total liver lipids were determined and liver weights were recorded in groups of six rats fed dietary levels of 300 or 600 ppm EDC for 5 wk or 1600 ppm for 7 wk and hepatic triglyceride levels were also measured in the rats fed 1600 ppm.

Long-term study

Growth experiments. The rats used were bred locally, 90 male and 90 female litter mates being divided into five equal groups, with 18 animals of each sex per group. Males and females were housed separately in stainless-steel cages each holding six animals. The experiment was started about 2 wk after weaning. Feed consumption and weight gain were recorded weekly up to wk 13 and in every second week thereafter.

Reproduction tests. After 6 wk on the experimental diets, the 3-month-old females were mated with untreated males for a preliminary test of their reproductive capacity. Thereafter, at about 2-monthly intervals, 45 experimental males were used to breed all the females, in cages housing one male and two females for 10 days. In order to give the remaining 45 males an opportunity to mate, they were housed with hysterotomized (normally cycling but sterile) females. The control diet was fed during these 10-day periods. Males mated with treated females in one trial were switched to the hysterotomized females in the next one, and *vice versa*. This alteration allowed the reasonably accurate elimination of infertile males and females after four trials.

Following mating, the experimental animals were returned to their original cages. Females were weighed twice a week and when they had gained 60 g they were placed in individual cages until parturition. At parturition, litter size and weight were recorded. After 10 days with the dams, the pups were again counted, the litters were weighed, and the dams were returned to the communal cages.

Biochemical tests. Total liver fat was determined by the method of Folch, Lees & Sloane Stanley (1957), involving homogenization of 3-g liver samples with 20 vols chloroform-methanol (2:1, v/v), filtration and evaporation of the solvent in a rotary evaporator, after which the fat was dried at 60°C to constant weight. For triglyceride determinations, the extracted fat was dissolved in 25 ml chloroform and aliquots of this solution were treated by the method of Van Handel & Zilversmit (1957). Phospholipids were determined after wet digestion by the colorimetric method for phosphorus (Gomori, 1942).

For serum analyses, the animals were killed by decapitation, blood was collected and left to coagulate, and serum was obtained after centrifugation for 20 min at 5000 rev/min in a Servall centrifuge at 5°C. Most of the tests were performed on the same day, and some of them on the next day, if the procedure allowed storage in a refrigerator. Total protein was determined by the biuret reaction (Weichselbaum, 1946), albumin by the procedure using bromcresol green, described in Technical Bulletin No. 11 (British Association of Clinical Biochemists, Scientific and Technical Committee), glucose and urea according to the Technicon Autoanalyzer method (Technicon Instrument Corp., Method File N-16 b), cholesterol by the Lieberman-Burchardt reaction (Rappaport & Eichhorn, 1960), uric acid with Folin reagent according to the modification of Eichhorn, Zelmanowski, Lew, Rutenberg & Fanias (1961) and transaminases according to Reitman & Frankel (1957).

Statistical evaluation. All results were subjected to analysis of variance, and the multiple range test of Duncan (1955) was used to evaluate treatment means.

RESULTS

Preliminary studies

The growth rate of the male rats fed mash containing 150 or 275 ppm CCl_4 did not differ significantly from that of the controls. The average total weight gain of male rats after 6 wk on trial was 175·3, 176·0, 161·8 and 152·9 g for control and ascending CCl_4 levels, respectively. The highest CCl_4 level, 520 ppm, depressed weight gain significantly ($P < 0·05$) compared with the other groups. The corresponding values for females after 5 wk on trial were 92, 94, 94 and 92 g, the weight gain being clearly unaffected by the treatment.

The most characteristic sign of CCl_4 poisoning was evident in the liver, in which total lipids and triglyceride levels were significantly higher in the CCl_4-treated animals, except the group fed 150 ppm, than in the controls. The levels of phospholipids were unchanged (Table 1). The calculated amount of CCl_4 consumed

Table 1. *Determination of fat in the livers of male and female rats fed for 6 and 5 wk, respectively, on diets containing 0–520 ppm CCl_4*

Dietary level (ppm)	Mean levels (mg/g liver, wet weight) of		
	Total fat	Triglycerides	Phospholipids
	Males		
0 (control)	61·0 ± 6·6	27·0 ± 3·4	
150	71·0 ± 6·0	25·0 ± 3·0	
275	136·0 ± 21·0**	53·4 ± 21·4**	
520	229·0 ± 49·0**	99·5 ± 16·8**	
	Females		
0	70·0 ± 8·2	7·0 ± 3·6	38·5 ± 4·0
150	62·0 ± 7·0	7·0 ± 3·0	35·9 ± 3·1
275	96·0 ± 7·0*	32·5 ± 7·7**	39·5 ± 0·8
520	174·0 ± 22·0**	73·5 ± 14·5**	39·3 ± 2·7

Values are means for groups of six rats and those marked with asterisks differ significantly from the control value: *$P < 0.05$; **$P < 0.01$.

daily by the rats given the high CCl_4 level approached 1% of the LD_{50} for this species (about 5 g/kg body weight).

The rats fed EDC in the mash did not show changes in liver fat with dietary levels of EDC below 1600 ppm (Table 2). With 1600 ppm, a fat accumulation of about 15% was noted, an increase much smaller than that induced by levels of CCl_4 several times lower.

The reproductive activity of male and female rats was not affected by either fumigant.

Long-term study

On the basis of the above preliminary experiments, the fumigant-residue levels chosen for 2-yr trials were 80 and 200 ppm for CCl_4 and 250 and 500 ppm for EDC. The rate of growth of the male and female rats is recorded in Table 3. No significant differences were found between the different groups either in growth or in feed consumption and feed efficiency.

The reproductive findings in the females are presented in Table 4. The difference between the high-CCl_4 group and the other groups in the number of parturitions was due to a very low parturition percentage in the fourth pregnancy. In the fifth pregnancy, the high-CCl_4 group returned to normal and the number of parturitions was higher than that in the control group. The difference may be regarded, therefore, as incidental. The size and weight of litters and the mortality rate of the pups were unaffected by the treatments.

In yr 2, the fertility of the females dropped steadily and after five pregnancies only a few females from each group conceived. Seven matings were conducted during the whole period. In the seventh, the females had to be assigned to young control males, due to high mortality among the experimental males at the end of the 2-yr trial.

The distribution of sterile males after four matings was normal, with one in the control, high-CCl_4 and low-EDC groups and two in the high-EDC and low-CCl_4 groups. The treatments had no effect on male fertility and, in general seemed to have no effect on the reproductive activity of the rats of either sex.

From the age of 14 months, all the animals began to suffer from chronic respiratory disease, and the mortality rate increased (Table 5). The females were more resistant to such illness than the males, but no effects of treatment were observed.

After 2 yr, surviving animals were killed and the biochemical tests for general metabolic and liver functions were carried out. The results (Table 6) showed no significant differences between the control and the treatment groups except for the serum-protein values, which were significantly ($P < 0.05$) higher in the male rats of the control and low-CCl_4 groups than in those of the other groups. The most pronounced difference was found in the globulin fraction, indicating a more

Table 2. *Weight and fat content of the livers of rats fed EDC in the diet at levels of 300–600 ppm for 5 wk (Trial 1) or 1600 ppm for 7 wk (Trial 2)*

Dietary level (ppm)	Liver weight (g/100 g body weight)		Liver content (mg/g wet liver) of		
			Total fat		Triglycerides
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 2
0 (control)	3·89	3·41	66·0	67·0	16
300	3·93		63·0		
600	3·81		65·0		
1600		3·53		76·0*	28*

Values are means for groups of six rats and those marked with an asterisk differ significantly ($P < 0.05$) from the control value.

Table 3. *Mean body weights of male and female rats fed mash fumigated with EDC or CCl_4*

Duration of treatment (wk)	Body weight (g) of rats fed diet containing				
	No fumigant (control)	CCl_4 (ppm)		EDC (ppm)	
		80	200	250	500
	Males				
0	130	131	130	131	130
1	160	160	158	160	159
2	192	190	184	186	190
3	218	218	210	216	216
4	244	250	239	249	246
5	271	275	263	274	268
6	297	301	290	299	292
7	313	314	304	314	306
8	328	334	322	334	323
9	340	346	337	345	334
10	354	362	348	360	354
11	362	366	356	367	362
12	370	373	362	376	368
13	376	380	367	384	376
	Females				
0	110	110	110	110	110
1	133	133	130	133	132
2	153	151	152	155	152
3	170	169	169	170	170
4	189	186	186	188	187
5	202	201	197	202	199

advanced stage of chronic disease in these than in the other animals. This was confirmed by a post-mortem examination of the respiratory system.

The biochemical tests did not reveal any effect either on liver function, as indicated by transaminases and cholesterol values, or on kidney function, as shown by the urea and uric acid levels. In addition to the values given in Table 6, serum levels of chloride, sodium and potassium were determined and found to be (in mequiv./litre): 100 and 103, 142 and

Table 4. *Reproductive performance of female rats fed mash fumigated with EDC or CCl_4*

Parameter	Values for groups fed diet containing				
	No fumigant (control)	CCl_4 (ppm)		EDC (ppm)	
		80	200	250	500
	Pregnancy 1				
No. of females mated	18	18	18	18	18
No. pregnant (% of total)	83	89	72	83	100
No. with litters (% of total)	72	89	67	83	100
Mean litter size	10·3	12	11·8	11·3	11·7
Mortality of young (%):					
at birth	16·4	0	8·5	0	0·9
at weaning	6·0	11·4	9·2	8·9	9·9
Mean body weight (g) of young:					
at birth	5·4	5·1	5·0	5·6	5·4
at weaning	14·5	13·0	13·3	13·7	14·3
	Pregnancies 2–5				
No. of females mated	66	72	72	72	72
No. pregnant (% of total)	58	70	56	72	67
No. with litters (% of total)*	53	64	44	71	64
Mean litter size	9·4	9·2	9·8	8·8	9·8
Mortality of young (%) at 10 days	9·8	23	11·3	11·1	13·7
Mean body weight (g) of young:					
at birth	5·7	5·5	5·4	5·5	5·5
at weaning	14·2	15·9	14·3	15·9	15·5

* No. of females producing litters declined sharply in pregnancies 4 and 5 (i.e. in females more than 1 yr old).

Table 5. *Survival of male and female rats fed mash fumigated with EDC or CCl_4 in a 2-yr feeding study*

Duration of study (months)	No. of survivors in groups fed diet containing									
	No fumigant (control)		CCl_4 (ppm)				EDC (ppm)			
			80		200		250		500	
	M	F	M	F	M	F	M	F	M	F
0	18	18	18	18	18	18	18	18	18	18
9	17	17	18	18	18	18	17	18	18	18
12	16	16	17	18	17	18	15	18	15	18
15	16	16	16	17	16	18	13	18	14	18
18	15	15	15	16	11	16	10	17	12	18
21	7	12	8	10	6	10	3	12	3	14
24	4	9	3	5	4	7	3	12	2	10

145, and 6·7 and 7·6 respectively, in females and males of all groups. No fatty livers were detected in the treated animals.

It seems that the levels of fumigants used did not affect the growth, health or reproductive activity of the male or female rats.

DISCUSSION

The difficulty of dealing with volatile compounds in the mash was overcome by finding storage conditions that caused minimal changes in fumigant levels and by training the animals to eat the fumigated mash during a relatively short period of time. Although there are some approximations in calculating the amounts of residue consumed, the methods seem suitable for the purpose of establishing the no-effect levels of fumigant for the experimental animals. The calculation is as follows: the maximal level of CCl_4 fed to rats without harmful effect during the 2-yr trial was 200 ppm; young and adult rats consumed daily from 10 to 30 g feed, according to age, and since they weighed from about 100 g at the beginning to about 400 g at the end of the experiment, it can be calculated that the CCl_4 dose consumed with the high-CCl_4 diet ranged from 15 to 25 mg/kg body weight; as only about 70% of the dose was actually consumed because of desorption of the residues during the eating period, the rats ate from 10 to 18 mg CCl_4/kg body weight. According to criteria of growth, sexual development, reproductive activity and general clinical symptoms, these levels were completely safe. The acceptable daily intake and tolerance proposed, therefore, are 10 mg/kg body weight and 100 ppm in the diet, respectively.

Table 6. *Terminal serum analyses in rats fed mash fumigated with EDC or CCl_4 for 2 yr*

Serum component	Mean values* for groups fed diet containing				
	No fumigant (control)	CCl_4(ppm)		EDC (ppm)	
		80	200	250	500
	Males				
Glucose (mg/100 ml)	128·0 ± 15·1	139·0 ± 23·0	102·8 ± 6·5	94·3 ± 12·6	109·0 ± 17·0
Protein (g/100 ml)	7·6 ± 0·09	7·5 ± 0·5	7·1 ± 0·13	6·9 ± 0·35	7·0 ± 0·05
Albumin (g/100 ml)	3·5 ± 0·06	3·5 ± 0	3·3 ± 0·06	3·0 ± 0·2	3·2 ± 0
Globulin (g/100 ml)	4·1 ± 0·12	4·0 ± 0·5	3·7 ± 0·11	3·85 ± 0·55	3·8 ± 0
Urea (mg/100 ml)	46·5 ± 4·0	48·5 ± 7·5	45·8 ± 3·6	50·0 ± 5.9	42·5 ± 5.5
Uric acid (mg/100 ml)	1·37 ± 0·06	1·34 ± 0·05	1·48 ± 0·06	1·31 ± 0·05	1·15 ± 0·05
Cholesterol (mg/100 ml)	103·3 ± 3·3	166·5 ± 20·5	115·7 ± 12·1	149·3 ± 12·5	131·8 ± 31·8
GOT (IU)	103·8 ± 12·8	100·5 ± 10·5	109·3 ± 13·4	89·0 ± 13·0	69·5 ± 8·5
GPT (IU)	20·0 ± 2·9	22·5 ± 2·5	19·0 ± 2·9	16·7 ± 1·7	22·5 ± 2·5
	Females				
Glucose (mg/100 ml)	97·5 ± 5·0	89·7 ± 2·3	94·6 ± 3·6	91·8 ± 7·9	100·8 ± 9·0
Protein (g/100 ml)	6·9 ± 0·30	7·6 ± 0·34	7·0 ± 0·18	7·2 ± 0·40	7·0 ± 0·36
Albumin (g/100 ml)	3·8 ± 0·35	3·5 ± 0·35	3·6 ± 0·09	3·2 ± 0·23	3·5 ± 0·20
Globulin (g/100 ml)	3·1 ± 0·33	4·0 ± 0·18	3·4 ± 0·17	4·0 ± 0·39	3·3 ± 0·40
Urea (mg/100 ml)	43·8 ± 5·3	50·6 ± 5·5	43·4 ± 1·5	47·0 ± 1·9	45·8 ± 5·4
Uric acid (mg/100 ml)	1·60 ± 0·11	1·87 ± 0·29	1·47 ± 0·22	1·45 ± 0·05	1·60 ± 0·09
Cholesterol (mg/100 ml)	87·3 ± 9·6	65·6 ± 4.7	86·7 ± 9·4	76·5 ± 16·0	103·0 ± 20·7
GOT (IU)	119·3 ± 26·0	145·0 ± 25·4	158·6 ± 25·4	123·8 ± 20·2	115·8 ± 18·3
GPT (IU)	15·9 ± 3·6	20·5 ± 0·5	25·8 ± 3·3	14·1 ± 2·4	15·0 ± 0.5

GOT = Glutamic-oxalacetic transaminase GPT = Glutamic-pyruvic transaminase

* Means ± SEM for groups of three or four males and four or five females.

The best proof of the reliability of the method seems to be the appearance of fatty livers after 5–6 wk on diets containing 275 ppm CCl_4. Rats on this diet consumed about 40 mg/kg body weight daily, an amount close to 1% of the LD_{50}.

Osumi, Amano & Shimamoto (1967) administered CCl_4 to rats orally twice weekly at doses from 38 to 200 mg/kg body weight. The calculated daily dose given to these rats was in the range used in the present feeding experiments. After 8 wk, the rats developed dose-dependent fatty livers and, at high levels, histochemical changes were observed in the livers.

The proposed acceptable daily intake and tolerance for EDC, calculated on the same basis as those for CCl_4, are 25 mg/kg body weight and 250 ppm, respectively.

Kenaga *et al.* (1969) calculated the safe residues of fumigants in food from the threshold limit values, the atmospheric concentrations considered safe for industrial workers to breathe for many years. Using a safety factor of 10, they calculated that the safe level, based on a 60-kg man consuming 400 g cereal products/day, was 49 ppm for CCl_4 and 150 ppm for EDC. The respective acceptable daily intakes were 0·33 and 1 mg/kg body weight.

We propose a tolerance of 10 ppm for CCl_4 and EDC in human food. The daily intake, calculated on the above basis, will be 0·07 mg/kg body weight, which is about 100 times less than the acceptable daily intake for animals (see also Alumot, Meidler, Holstein & Herzberg, 1976). A safety factor of 100 is usually applied to laboratory-animal data. A level of 10 ppm of fumigant is not likely to occur in cereal foods for human consumption, since these are generally cooked or baked. The residue level in bread baked from fumigated wheat containing 10 ppm CCl_4 was less than 0·5 ppm (Bondi & Alumot, 1974). Similar results were reported by Dutch scientists (Wit, Besemer, Das, Goedkoop, Loosjes & Meppelink, 1969). The normal fumigation practice seems to be a harmless procedure for man.

Olson, Haberman, Weisburger, Ward & Weisburger (1973) induced stomach cancer in mice and rats with EDB and dibromochloropropane. The drastic conditions of their experiments (frequent doses close to the LD_{50}) are representative of acute poisoning. These results are not relevant to our work, which deals with chronic administration of fumigant residues in feed.

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TOLERANCE AND ACCEPTABLE DAILY INTAKE OF ETHYLENE DICHLORIDE IN THE CHICKEN DIET*

E. ALUMOT, M. MEIDLER and P. HOLSTEIN

Agricultural Research Organization, The Volcani Center, Bet Dagan,

and

M. HERZBERG

Ichilov Hospital, Tel Aviv, Israel

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Abstract—Leghorn chickens of both sexes were fed mash fumigated with ethylene dichloride (1,2-dichloroethane) for 2 yr. Growth, performance of layers, fertility, reproduction and biochemical tests served as criteria for establishing the no-effect levels. Ethylene dichloride did not affect the growth, semen characteristics or fertility of the chickens, and the results of serum analyses on treated chickens did not differ from those on controls. Both fumigant levels, 250 and 500 ppm in the mash, caused a moderate but persistent decrease in egg weight from month 4 of the laying period. Egg production was affected only by the higher level of fumigant. A tolerance of 100 ppm and an acceptable daily intake of 5 mg/kg body weight seem to be justified for laying hens. For growing chicks and cocks the tolerance and acceptable daily intake are the same as those previously determined for rats, namely 250 ppm and 25 mg/kg, respectively.

INTRODUCTION

The importance of studying the effect of the prolonged feeding of animals on diets containing fumigant residues has already been emphasized (Alumot, Nachtomi, Mandel, Holstein, Bondi & Herzberg, 1976).

The use of chickens as experimental animals for establishing no-effect levels for man is justified by species differences in the long-term toxicity of fumigants. As mentioned in the introduction to the preceding report (Alumot *et al.* 1976), ethylene dibromide affected such different phenomena as laying activity in hens and spermatogenesis in bulls when administered in both cases at a level of about 1% of the LD_{50} daily for several weeks. On the other hand, reproductive activity in rats, the most widely used species for evaluation of toxicity, was not affected even by larger doses of ethylene dibromide (E. Alumot *et al.* unpublished data). Freerksen & Kazda (1974) proposed recently that the reproductive activity of the laying hen could provide a very sensitive test for the evaluation of drug activity.

The present report deals with observations on chickens fed for 2 yr on diets fumigated with two levels of ethylene dichloride (EDC). EDC is less toxic than ethylene dibromide and was therefore tried in the mash at higher levels than ethylene dibromide.

Carbon tetrachloride could not be tested on chickens as these animals are insensitive to both acute (Hall & Shillinger, 1923) and long-term administration (E. Alumot *et al.* unpublished data) of this compound.

EXPERIMENTAL

The fumigation of the mash, storage conditions and residue determinations, as well as the biochemical tests have been described (Alumot *et al.* 1976). The EDC levels in the mash were 250 ± 30 ppm and 500 ± 40 ppm.

Feeding procedure. Growing birds were fed three meals each day, from 06.30 to 08.30 hr, from 11.00 to 12.00 hr and from 14.00 to 16.00 hr. This regime was chosen after preliminary tests lasting 1 wk, during which the consumption was determined by weighing the mash in the troughs each hour. The controlled feeding resulted in a total consumption no lower than that by continuously fed animals. All the groups consumed the same amount of feed, so in practice paired-feeding was attained by this feeding regime. Laying hens were fed for two 2-hr periods during the day, and a third weighed portion was left overnight to ensure availability of food during egg formation. The accuracy of the paired feeding was thus interfered with during this third feeding. In preliminary experiments, the mash was tested for fumigant loss during the different feeding periods. The losses were about 20% after 1 hr in the feed trough and up to 40% after 2 hr. The average amount of EDC consumed was about 70% of the initial value.

Experimental design and conduct. White Leghorn chicks were divided into three groups, each consisting of ten males and 20 females, and fed control mash or fumigated mash containing either 250 or 500 ppm

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Table 1. *Growth and feed efficiency of Leghorn chicks fed EDC-fumigated mash*

Duration of trial (wk)	Values for chicks fed EDC levels (ppm) of					
	0		250		500	
	Weight (g)	Feed efficiency*	Weight (g)	Feed efficiency*	Weight (g)	Feed efficiency*
0	73	—	70	—	71	—
1	115	0·47	111	0·52	116	0·54
2	176	0·58	165	0·56	170	0·54
3	245	0·47	233	0·45	240	0·48
5	403	0·37	400	0·38	399	0·37
6	520	0·32	507	0·29	513	0·31
8	739	0·29	718	0·26	706	0·24

*Feed efficiency = (Weight gain/Food consumption)
Values are means for groups consisting of ten males and 20 females.

EDC. Growth and feed consumption were recorded weekly up to 8 wk of age and monthly thereafter.

At the age of 4·5 months, pullets were separated from cockerels and transferred to the laying house. After laying began, the number of pullets was reduced to 16/group by random selection. The pullets were placed in individual laying cages and their performance was recorded daily. Initially, eggs were weighed weekly on a group basis. When the first signs of weight decrease were noted, all the eggs were weighed individually for 1 wk. Thereafter, individual weighing was carried out on three consecutive days, twice each month.

The males were checked for semen production and sperm motility 12 times, twice weekly, when 7–8·5 months old. At the age of 8·5 months, seven cocks from each of the treated groups and five control cocks were placed in individual cages. Semen examination was continued.

The fertility of the cocks and the hens was tested in two trials by means of artificial insemination. In the first trial, half of the hens on the control and high-EDC diets were inseminated with semen obtained from control cocks and the remaining birds in each group with the semen of cocks on the high-EDC diet. In the second trial, the arrangement was reversed. Eggs were collected on days 2–7 after insemination and checked for the presence of embryos after incubation for 4 days. In a separate experiment, several hens from the control and two EDC-treated groups were inseminated with semen from control or EDC-treated cocks. The eggs were collected and incubated for 3 wk under standard conditions of temperature and humidity in a laboratory incubator.

Table 2. *Flock production rate during months 1–3 of laying by Leghorn hens fed EDC-fumigated mash*

Duration of laying period (months)	Flock production rate (%) for groups fed EDC levels (ppm) of		
	0	250	500
1	90·1	86·0	84·7
2	87·1	75·5	84·5
3	82·0	75·7	83·7

At the end of the 2-yr feeding period, blood samples were taken from all the hens for serum analyses (for methods, see Alumot *et al.* 1976).

All the results were subjected to analysis of variance, and the multiple range test of Duncan (1955) was used to evaluate treatment means.

RESULTS

Records of growth and feed efficiency during the first 8 wk of the trial (Table 1) showed no differences between the control and EDC-treated groups. Subsequently the chicks were weighed monthly, and at 4·5 months of age, when the sexes were separated, there was again no significant difference between treatments. The average female weights were 1376 ± 85, 1385 ± 77 and 1363 ± 68 g in the control, high-EDC and low-EDC groups, respectively, while the corresponding weights of the males were 1875 ± 108; 1820 ± 95 and 1820 ± 111 g.

The performance of the laying hens was not affected by EDC treatment during the first 3 months of full laying activity (Table 2).

A highly significant difference in egg weights between the control group and the two EDC-treated groups appeared in month 3 of full laying activity (Table 3) and persisted up to the end of the 2-yr trial (Table 4).

Table 3. *Mean weight of eggs from groups of Leghorn hens fed EDC-fumigated mash*

Duration of laying (wk)	Egg weights (g) for groups fed EDC levels (ppm) of		
	0	250	500
8	60·3	61·5	60·0
9	62·8	59·6	60·4
10	60·7	59·7	58·3
11	58·7	58·3	57·0
12	61·0	58·0*	57·3*

Values are means for eggs laid during each week by groups of 15 or 16 hens, and those marked with an asterisk differ significantly from the control value (*$P < 0·01$).

Table 4. *Mean egg weight determined individually twice each month on three consecutive days for groups of Leghorn hens fed EDC-fumigated mash*

Duration of laying (months)	Mean egg weights (g) for groups fed EDC levels (ppm) of		
	0	250	500
4	58·8 ± 3·2 (15)	55·7 ± 4·9 (16)	55·6 ± 4·1 (15)
7	63·8 ± 3·8 (15)	58·0 ± 5·4 (15)	55·7 ± 3·7 (14)
11	67·4 ± 5·0 (15)	61·1 ± 5·9 (14)	60·5 ± 4·8 (12)
12	66·9 ± 4·2 (14)	60·3 ± 5·2 (14)	60·3 ± 4·4 (12)
13	65·9 ± 4·8 (14)	60·8 ± 5·3 (14)	60·0 ± 4·1 (12)
14	66·1 ± 5·3 (14)	60·4 ± 5·4 (14)	59·1 ± 3·6 (12)
15	65·1 ± 4·9 (14)	59·6 ± 6·1 (14)	58·9 ± 3·1 (12)
16	64·5 ± 5·0 (14)	59·1 ± 6·7 (14)	57·8 ± 3·3 (12)
17	63·4 ± 5·7 (14)	57·9 ± 5·1 (14)	58·6 ± 3·8 (12)
18	64·3 ± 5·9 (14)	59·8 ± 5·8 (14)	58·4 ± 4·1 (12)

Values are expressed as the means ± SD for the numbers of hens indicated in parenthesis and all those for EDC-treated hens were significantly lower ($P < 0.01$) than the corresponding control figure.

The few deaths that occurred during the experiment (Table 4) were caused by leucosis (one hen from each group) at the beginning of the laying period and by reproductive disorders (a total of five hens) at periods indicated in the table.

From month 4 of laying, the performance of hens on the high-EDC diet, but not of those on the lower, dropped significantly ($P < 0.05$) in comparison with that of the control group (Table 5). The lower production rate of the hens fed 500 ppm EDC in the mash was caused by the lowering both of the individual laying rate and of the number of layers in the group.

The feed intake of the high-EDC group was lower than that of the other groups, particularly in the period after moulting was induced (Table 6). It is possible that the reduced intake was a consequence of the lower egg production.

In contrast to the females, the males were not affected by EDC treatment. No difference occurred between cocks of the control and EDC groups as far as semen production and sperm motility were concerned. The number of poor semen producers was similar in all groups (three or four/group). These cocks were not used in the fertility tests.

The results of the two fertility trials were as follows: control cocks and EDC hens, 100% fertile eggs; control cocks and control hens, 97%; EDC cocks and EDC hens, 100%; and control hens and EDC cocks, 87%. No significant differences were found in the fertilization rates following insemination with the control- or EDC-group semen, regardless of whether the hens were from the control or EDC group. Hatchability (% of fertile eggs) was 85·7, 94·1 and 96·8 in the control, low-EDC and high-EDC groups, respectively, re-

Table 5. *Egg-production by EDC-treated Leghorn hens from month 4 of laying to the end of the trial*

Duration of laying (months)	Flock production rate, %			Layers' production rate, %			Laying hens, % of total		
		EDC			EDC			EDC	
	Control	250 ppm	500 ppm	Control	250 ppm	500 ppm	Control	250 ppm	500 ppm
4	83·6	74·5	64·5*	83·6	74·5	69·0*	100	100	93
5	75·5	73·6	57·4*	75·5	73·6	66·2*	100	100	87
6	68·3	67·4	46·7*	68·3	67·4	59·4*	100	100	79
7	61·6	51·0*	50·0*	61·6	55·9	58·3	100	73	86
8†	49·3	36·0	21·3*	49·3	42·0	25·6*	100	86	83
10	21·0	27·7	8·0*	26·3	35·3	16·0*	80	79	50
11	63·1	54·3	47·1*	63·1	58·5	56·5	100	93	83
12	72·9	79·7	62·9*	78·1	79·7	68·6*	93	100	92
13	76·6	74·7	68·7	82·1	74·7	74·9	93	100	92
14	68·4	71·6	54·2*	73·3	71·6	59·1*	93	100	92
15	58·0	63·0	46·8*	62·5	63·0	57·7	93	100	83
16	59·0	60·8	48·4*	63·5	60·8	58·1	93	100	83
17	53·6	48·9	41·3*	57·7	57·3	49·6*	93	86	83
18	50·0	50·0	41·0*	63·6	58·3	55·2	79	86	75

†In month 8 (November 1972) all the hens were subjected to forced moulting by feed withdrawal in order to renew the production for the second year. No eggs were laid in December, and laying began again in January 1973.

Values marked with an asterisk differ significantly ($P < 0.05$) from the control, on the basis of analysis of the individual production rate.

Table 6. *Effect of EDC fumigation of mash on monthly feed consumption by laying hens*

Duration of laying (months)	Feed consumption (kg mash/hen/month) by groups fed EDC levels (ppm) of		
	0	250	500
3	2·9	2·6	2·8
4	3·5	3·3	3·3
5	2·8	2·7	2·8
6	2·6	2·3	2·3
7	2·8	2·7	2·7
8*	1·6	1·7	1·6
9	2·7	2·5	2·1
10	2·7	2·7	2·3
11	3·0	3·0	2·3
12	3·2	3·1	2·7
13	3·9	3·6	3·3
14	3·0	2·8	2·6
15	2·9	2·7	2·6
16	3·6	3·2	3·0
17	2·8	2·6	2·2
18	3·4	3·3	2·8
Total	47·4	44·8	41·4

*Food restricted to induce forced moulting (see footnote (†) to Table 5.

gardless of the semen source, and the respective average chick weight was 41·2. 40·0 and 39·6 g. The differences were not statistically significant.

At the end of the 2-yr trial, blood samples from all the hens were analysed for biochemical parameters of liver function and general health (Table 7). No significant differences were found between control and treated hens.

Table 7. *Terminal analyses of serum from laying hens fed EDC-fumigated mash for 2 yr*

Serum component	Mean values for groups fed EDC levels (ppm) of	
	0	500
Glucose (mg/100 ml)	220·7 ± 9·4	207·8 ± 7·2
Protein (g/100 ml)	6·1 ± 0·3	6·3 ± 0·4
Albumin (g/100 ml)	2·1 ± 0·13	2·2 ± 0·09
Globulin (g/100 ml)	4·0 ± 0·37	4·1 ± 0·35
Uric acid (mg/100 ml)	5·2 ± 0·5	5·3 ± 0·4
Cholesterol (mg/100 ml)	200·8 ± 21·5	240·0 ± 32·8
GOT (IU)	106·0 ± 6·3	105·3 ± 9·9
GPT (IU)	4·3 ± 1·1	4·2 ± 1·1

GOT = Glutamic–oxalacetic transaminase
GPT = Glutamic–pyruvic transaminase
Values are means ± SEM for groups of ten hens.

DISCUSSION

It is clear from the results reported here that neither EDC level fed in the mash influenced the growth or sexual development of male or female chicks. However, the 500-ppm level affected the feed intake and performance (number and weight of eggs) of the laying hens. At the lower level (250 ppm), egg weight but not egg number was significantly affected. Since the difference in egg weight between both EDC groups and the control was similar (about 5–6 g) and did not change during more than a year of laying, the effect of EDC on production may be regarded as relatively moderate. Ethylene dibromide, a chemically related compound, affected egg size drastically, causing a continuous drop in weight up to cessation of laying even at a level of 20 ppm in the mash (Bondi *et al.* 1955).

In contrast to the action of ethylene dibromide (Alumot, Nachtomi, Kempenich-Pinto, Mandel & Schindler, 1968), EDC did not affect the fertility of laying hens. The effect of the low EDC level was restricted to a moderate but constant depression in egg weight. To be on the safe side, therefore, we suggest that the tolerance for hens should be no more than 100 ppm EDC. The acceptable daily intake, based on a mash consumption of 100 g and a body weight of 2 kg is then about 5 mg/kg body weight. For growing chicks the tolerance and the acceptable daily intake are, as for rats, 250 ppm and 25 mg/kg body weight, respectively.

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POTATO STARCH AND CAECAL HYPERTROPHY IN THE RAT

E. A. EL-HARITH, J. W. T. DICKERSON and R. WALKER

Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, England

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Abstract—Raw and milled potato starch caused caecal enlargement of up to 800% when fed to rats as the sole carbohydrate source in synthetic diets containing 16–24% protein and 71–63% carbohydrate. Control animals received maize starch in the diet. Caecal enlargement was dose-related. The hypertrophy was reversible and caeca approached control size within 11 days of the replacement of potato starch by maize starch. Some deaths occurred in groups of animals receiving raw potato starch at dietary levels of 30% or more. A total of 21% of the animals receiving these levels of raw potato starch died, while no deaths were observed in control animals. There were no significant differences between test and control animals in potassium concentrations and osmolalities of caecal contents, or in serum electrolyte and urea concentrations. Activities of serum glutamic–pyruvic transaminase and alkaline phosphatase were significantly higher in test animals but the histology of the liver, gastrointestinal tract and kidney were apparently normal. Caecal distension resulted in pressure on the diaphragm and impaired respiratory function, as indicated by a higher arterial blood pCO_2 and lower pO_2 and an increase in lactate concentration in venous blood. It is suggested that impaired respiratory function may have played an important role in the deaths associated with dietary potato starch and caecal enlargement.

INTRODUCTION

Several natural food components and food additives cause caecel enlargement when fed to rats. These compounds include some raw starches (Jelinek, Katayama & Harper, 1952), modified starches (de Groot, Til, Feron, Dreef-van der Meulen & Willems, 1974; Joint FAO/WHO Expert Committee on Food Additives, 1972), lactose (Reussner, Andros & Thiessen, 1963), sorbitol (Morgan & Yudkin, 1957), maltitol (Hosoya, 1972) and a high-molecular-weight glucose-syrup fraction (Birch & Etheridge, 1973). Similar effects have been observed with polyethylene glycol (Loeschke, Uhlich & Halbach, 1973) and magnesium sulphate (Moinuddin & Lee, 1960). In the safety evaluation of food additives, caecal enlargement *per se* without other toxicological sequelae poses a problem of interpretation, but it has been suggested that it results from an increase in the bulk of the caecal contents (Joint FAO/WHO Expert Committee on Food Additives, 1972) or is a process of physiological adaptation controlled by the osmolality of the caecal contents (Leegwater, de Groot, & van Kalmthout-Kuyper, 1974). In either case it might be concluded that the observed effects on the gastrointestinal tract were without toxicological significance. However, in our laboratory, the feeding to rats of raw potato starch as the sole carbohydrate source in synthetic diets led to gross caecal enlargement and a number of deaths within 3 wk (El Harith, Walker & Dickerson, 1975). These effects were not found with maize starch.

This paper describes the results of a further study of these effects.

EXPERIMENTAL

Animals. Weanling male Wistar albino rats (body weight 40–50 g) of an SPF-derived inbred colony were used for all experiments.

Materials and methods. Raw maize and potato starches were obtained from BDH Ltd. Poole, Dorset. Milled potato starch was prepared by milling raw potato starch in a laboratory ball-mill with porcelain balls for 4 hr. Autoclaved potato starch was prepared by moistening the raw starch with water, heating it in an autoclave at 120°C for 2 hr and then drying at 40°C for 24 hr. 'Caecal fluid' was obtained by homogenizing caecal contents with deionized water (1:1, w/w). Electrolyte concentrations were determined in caecal fluid and blood serum, sodium and potassium in an EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex) and chloride by the method of Schales & Schales (1941). Osmolalities were determined with the Halbmicro-Knauer automatic osmometer.

Serum alkaline phosphatase, glumatic–pyruvic transaminase, urea and blood lactate were determined colorimetrically using Boehringer Biochemica Test combinations, the tests being based on the original methods by Bessey, Lowry & Brock (1946), Reitman & Frankel (1957), Fawcett & Scott (1960) and Hohorst (1962), respectively. Blood pH and gases in arterial blood samples collected from carotid cannu-

lae were determined with the IL-ultramicro pH/blood gas analyser, model 113-S1.

Experimental protocols

The animals were housed singly in cages with raised screen floors. Environmental conditions were controlled at a temperature of 20 ± 2°C and a relative humidity of 25 ± 5%, with a 12-hr alternate light/dark cycle. Food and water were available *ad lib.* and food intake, body weight and faecal weight were recorded weekly, or more frequently as indicated below.

Experiment 1. In a preliminary experiment, five groups of five animals were maintained for 3 wk on a diet with the following basic composition: 71% carbohydrate, 16% protein (casein), 5% corn oil (Saladin brand, Craigmillar Ltd., London S.E.6), 4% vitamin mix (Cooper's Nutritional Products, Witham, Essex) and 4% mineral mix (Cox Ltd., Brighton, Sussex). The carbohydrate source was varied between the groups, group 1A being given maize starch, 1B1 raw potato starch, 1B2 raw potato starch on days 1–10 and maize starch on days 11–21, 1C ground potato starch and 1D autoclaved potato starch. Food intakes and body weights were monitored daily. After 3 wk the animals were killed by asphyxiation with CO_2 and autopsied. The gastro-intestinal tract was dissected and the different parts were weighed. The liver, kidneys, adrenals and thymus were also removed and weighed. Sections of the gastro-intestinal tract and of the organs mentioned were fixed in buffered formol-saline. The tissues were then dehydrated with ethanol, embedded in paraffin wax, cut into 5 μm sections, mounted and stained with eosin–haematoxylin by standard histological procedures (Drury & Wallington, 1967).

Experiment 2. To determine the dietary level of potato starch necessary to produce caecal enlargement, five groups of five rats were fed diets of similar composition to those used in experiment 1, except that the carbohydrate source was composed of the following percentages of maize and raw potato starch, respectively: 2A 71 and 0%, 2B 55 and 16%, 2C 41 and 30%, 2D 21 and 50% and 2E 0 and 71%. Food intakes and body weights were again recorded weekly and after 3 wk the animals were killed and examined as in experiment 1.

Experiment 3. To investigate whether caecal enlargement was influenced by dietary protein concentrations, six groups of five animals were fed diets containing maize starch (A groups) or potato starch (B groups). In these synthetic diets the protein (casein) and carbohydrate percentages, respectively, were varied as follows: groups 3A1 and 3B1 16 and 71%, 3A2 and 3B2 20 and 67%, and 3A3 and 3B3 24 and 63%. Food intake and body weight were recorded weekly, and after 3 wk the animals were killed and autopsied as in previous experiments.

Experiment 4. This was designed to demonstrate the effects of potato starch on the gastro-intestinal tract and on other physiological parameters, with a view to elucidating the cause of occasional deaths in other experiments. Two groups of 40 rats were randomly selected and assigned to a control group (4A) and a test group (4B). The animals received a diet similar in composition to that used in experiment 1, with the control diet containing 71% maize starch and the test diet 71% raw potato starch. After 3 wk the animals were anaesthetized with Nembutal (20–35 mg/kg body weight) prior to collection of blood samples for analysis. The carotid artery was cannulated in ten animals from each group and 1 ml arterial blood was collected in a syringe for analysis of blood pH, pCO_2 and pO_2, while samples of blood from the inferior vena cava were collected by syringe for the determination of the lactate concentration in venous blood. The remaining 30 animals from each group were bled from the inferior vena cava by syringe for analysis of serum enzymes, urea and electrolytes. The animals were killed by exsanguination and the caeca were excised and weighed. Sections of caecum, gastro-intestinal tract, liver and kidney were fixed in formol-saline for subsequent processing and histological examination. Caecal contents were prepared for sodium, potassium and chloride analyses and for osmolality determinations.

RESULTS

Body and organ weights (experiment 1)

Animals receiving maize or autoclaved potato starch gained weight steadily throughout the experiment, whereas there was a check in weight gain in the other groups after 10–13 days, despite a relatively higher food intake. The terminal body weight minus gut weight was lower in groups 1B1 and 1C and higher in group 1D than in the control group 1A, while that for group 1B2 did not differ significantly from the control value (Table 1).

All the post-gastric sections of the intestinal tract were significantly enlarged in group 1B1 (Table 1) and the caecum and large intestine were significantly enlarged also in group 1C. In both of these groups, there was an increase in caecal tissue as well as in its contents, whereas animals in group 1B2 and 1D showed a significant increase only in the mass of caecal contents. Differences in the weights of other organs were small, but the liver weight of the animals on raw potato starch (group 1B1) was significantly heavier than that of the group receiving maize starch, and all the animals receiving raw or processed potato starch throughout had lower adrenal weights (Table 1).

Organ weights in group 1B2 did not differ significantly from those of the animals receiving maize-starch diet, but the caecal contents remained higher (Table 1).

Food efficiency values

Estimation of food efficiencies was complicated by the fact that there was gross caecal enlargement

Table 1. *Relative organ weights in rats fed diets containing 71% maize or potato starch for 3 wk*

Organ	Values for group				
	1A (m)	1B1 (rp)	1B2 (rp/m)	1C (gp)	1D (ap)
Body weight minus gut weight (g)	159·1 ± 6·3	147·1 ± 8·4*	157·2 ± 4·7	143·2 ± 6·9*	177·0 ± 8·1*
Organ weights (g/100 g body weight)					
Caecum	0·27 ± 0·05	1·45 ± 0·37***	0·33 ± 0·34	1·60 ± 0·85***	0·38 ± 0·09
Caecal contents	0·86 ± 0·21	6·70 ± 2·43***	1·44 ± 0·24**	9·95 ± 3·18***	2·70 ± 0·50***
Small intestine	1·99 ± 0·56	4·08 ± 1·10**	1·83 ± 0·35	2·75 ± 1·11	1·43 ± 0·21
Large intestine	0·38 ± 0·11	0·92 ± 0·29**	0·49 ± 0·09	0·81 ± 0·30*	0·37 ± 0·27
Stomach	0·59 ± 0·06	0·48 ± 0·08*	0·70 ± 0·11	0·58 ± 0·22	0·54 ± 0·06
Liver	5·69 ± 0·59	7·74 ± 0·56*	6·26 ± 0·41	4·99 ± 1·08	5·94 ± 0·12
Kidneys	0·97 ± 0·06	0·98 ± 0·16	1·00 ± 0·12	0·97 ± 0·11	0·87 ± 0·05
Thymus	0·45 ± 0·09	0·30 ± 0·10*	0·39 ± 0·03	0·32 ± 0·05*	0·36 ± 0·05
Adrenals	0·046 ± 0·000	0·030 ± 0·007**	0·043 ± 0·007	0·036 ± 0·005**	0·023 ± 0·008***

m = Maize starch rp = Raw potato starch rp/m = Raw potato starch (days 1–10), maize starch (days 11–21)
gp = Ground potato starch ap = Autoclaved potato starch

Values are means ± SEM for groups of five rats and those marked with asterisks differ significantly (Student's *t* test) from that of the control group 1A: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

(accompanied by fluid retention) in all test animals. Terminal correction for caecal weight enabled a more valid estimate of food efficiency to be made.

In experiment 1, corrected food efficiency values for groups 1B1 and 1C, receiving raw potato or ground potato starch, were significantly lower than that for the control group 1A fed maize starch but those for groups 1B2 (raw potato followed by maize) and 1D (autoclaved potato) were not significantly different from the control value (Table 2). On the other hand, the corrected food efficiency value for group 1C (ground potato) was not significantly different from that of group 1B1 (raw potato) whereas the value for group 1D (autoclaved potato) was significantly higher (Table 2).

In experiment 2, corrected food efficiency values for the test groups 2B, 2C and 2D were all significantly lower than that of the control group 2A (maize diet). There was a more or less steady decrease in food efficiency as the percentage of raw potato starch in the diet increased (Table 2).

In experiment 3, corrected food efficiency values were significantly lower in all the groups receiving raw potato starch, irrespective of the percentage of protein in the diet. However, as expected, with higher protein levels in the diet there were higher food efficiencies for both test and control animals. Again in experiment 4, the food efficiency for the test group 4B (raw potato) was significantly lower than that for the maize-fed control group 4A (Table 2).

Table 2. *Food efficiency values determined in wk 3 of administration of diets containing maize starch or various potato starches to groups of rats*

Group	Dietary concn (%) of starch	Food efficiency‡	
		Uncorrected	Corrected
Experiment 1			
1A	71 m	0·37 ± 0·00	0·35 ± 0·00
1B1	71 rp	0·29 ± 0·06	0·20 ± 0·08***
1B2	71 rp/m	0·35 ± 0·00	0·33 ± 0·00
1C	71 gp	0·28 ± 0·02	0·16 ± 0·02***
1D	71 ap	0·34 ± 0·03	0·31 ± 0·03†
Experiment 2			
2A	71 m + 0 rp	0·37 ± 0·03	0·35 ± 0·03
2B	55 m + 16 rp	0·32 ± 0·01	0·26 ± 0·01*
2C	41 m + 30 rp	0·35 ± 0·05	0·25 ± 0·04*
2D	21 m + 50 rp	0·24 ± 0·03	0·15 ± 0·04**
2E	0 m + 71 rp	(not determined)	--
Experiment 3			
3A1	16 c + 71 m	0·34 ± 0·00	0·31 ± 0·00
3B1	16 c + 71 rp	0·19 ± 0·05	0·09 ± 0·04***
3A2	20 c + 67 m	0·34 ± 0·00	0·32 ± 0·00
3B2	20 c + 67 rp	0·25 ± 0·01	0·15 ± 0·01***
3A3	24 c + 63 m	0·35 ± 0·01	0·33 ± 0·01
3B3	24 c + 63 rp	0·26 ± 0·02	0·17 ± 0·01***
Experiment 4			
4A	71 m	0·42 ± 0·03	0·40 ± 0·03
4B	71 rp	0·30 ± 0·03	0·20 ± 0·03**

m = Maize starch rp = Raw potato starch
rp/m = Raw potato starch (days 1–10), maize starch (days 11–20) gp = Ground potato starch
ap = Autoclaved potato starch c = Casein

‡ Food efficiency: uncorrected = weight gain (g)/food intake (g); corrected = weight gain − weight of caecum (g)/food intake (g).

Values are means ± SEM for groups of five (experiments 1, 2 and 3) or 40 (experiment 4) rats and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control (A group) value: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. The dagger indicates a value differing significantly from that for group 1B1: $†P < 0.05$.

Caecal enlargement and mortality

Gross caecal enlargement and meteorism was noticed in all test groups (Table 3), but was less pronounced in group 1B2 (potato followed by maize) and group 1D (autoclaved potato). The filled caecal weights of groups 1C (ground potato) and 1D (autoclaved potato) were significantly higher and significantly lower, respectively, than that of group 1B1 (raw potato). Abdominal swelling was accompanied by

Table 3. *Relative 'filled' caecal weights and dried faecal weights for groups of rats fed diets containing maize starch or various potato starches for 3 wk*

Group	Dietary concn (%) of starch	Caecal weight (g/100 g body weight)	Faecal weight‡ (g/rat/wk)
Experiment 1			
1A	71 m	1·03 ± 0·05	4·18 ± 0·22
1B1	71 rp	8·15 ± 0·37***	29·84 ± 2·50***
1B2	71 rp/m	1·77 ± 0·04***	3·96 ± 0·15
1C	71 gp	11·55 ± 0·83***†††	25·60 ± 5·58***†
1D	71 ap	3·0 ± 0·09**†††	6·38 ± 0·38†††
Experiment 2			
2A	71 m + 0 rp	2·09 ± 0·66	4·26 ± 0·09
2B	55 m + 16 rp	4·12 ± 0·88**	8·48 ± 0·15***
2C	41 m + 30 rp	6·92 ± 0·73***	4·40 ± 0·11***
2D	21 m + 50 rp	7·70 ± 1·13***	33·75 ± 4·11***
2E	0 m + 71 rp	10·91 ± 1·45***	32·75 ± 1·18***
Experiment 3			
3A1	16 c + 71 m	1·77 ± 0·07	3·50
3B1	16 c + 71 rp	8·25 ± 1·25***	40·70
3A2	20 c + 67 m	1·35 ± 0·28	3·50
3B2	20 c + 67 rp	8·01 ± 0·74***	27·40
3A3	24 c + 63 m	1·62 ± 0·09	3·80
3B3	24 c + 63 rp	6·83 ± 1·05***	23·70

m = Maize starch rp = Raw potato starch
rp/m = Raw potato starch (days 1–10), maize starch (days 11–20) gp = Ground potato starch
ap = Autoclaved potato starch c = Casein

‡ Measured in wk 3; faeces were air-dried in experiments 1 and 2 and oven-dried at 110°C for 20 hr in experiment 3. No *t* test was done on the results for the latter samples, for which mean values only are given.

Values are means ± SEM for groups of five rats and those differing significantly (Student's *t* test) from the control (A) values are marked with asterisks: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. Daggers indicate values differing significantly from those for group 1B1: $†††P < 0{\cdot}001$.

reduced spontaneous activity. At autopsy the animals with abdominal swelling had grossly enlarged caeca compared with controls; there was resultant pressure on the liver and diaphragm and the rib cage was distended (Fig. 1). Experiment 2 showed that relative caecal weight increased linearly with increased dietary raw potato starch (Fig. 2).

One animal from group 1C (ground potato) died on day 13 of the experiment; it had an impacted caecum with semi-solid contents and signs of haemorrhage into the caecum. In experiment 2, one animal from each of the test groups 2E, 2D and 2C died on day 8, 16 and 17, respectively, but no caecal haemorrhage was observed. One animal in group 3B2 (20% protein and 67% raw potato) died on day 20 and in experiment 4, 12 deaths were recorded in group 4B (raw potato) between days 10 and 20. Haemorrhage into the caecum was noted in three of these cases.

Faecal weights

The faecal weights (air-dried or oven-dried) of the groups receiving raw potato starch were between two and ten times as high as those of the corresponding control groups (A) receiving maize starch (Table 3). In experiment 1, the mean faecal weight of group 1B2 (raw potato followed by maize) did not differ significantly from that of the control group (1A), but those of groups 1C (ground potato) and group 1D (autoclaved potato) were 5 times and 1.5 times higher, respectively. Compared with the value for group 1B1, that for group 1C did not differ significantly but that for group 1D was significantly lower (Table 3). In all groups receiving raw or milled potato starch, intact starch granules were observable on microscopic examination of the faeces.

Caecal contents

In test animals the caecal contents were soft, and meteorism was observed in both the caecum and colon. The concentrations of sodium and chloride were lower than in the controls but potassium concentrations and osmolalities did not differ significantly between the groups (Table 4).

Clinical chemistry and histology

Serum glutamic–pyruvic transaminase and alkaline phosphatase were significantly higher in test animals, but blood urea and serum electrolyte concentrations were not significantly different from those in the controls (Table 4). Arterial blood pCO_2 was significantly higher in test animals and pO_2 significantly lower, but the blood pH was unaffected. Venous-blood lactate was significantly higher in test animals (Table 4).

Histologically, the tissues of the gastro-intestinal tract and other organs appeared normal. There was no indication that the feeding of raw or processed potato starch induced any morphological changes.

DISCUSSION

The results presented here confirm earlier observations that raw potato starch caused caecal hypertrophy at levels as low as 16% in the diet. The magnitude

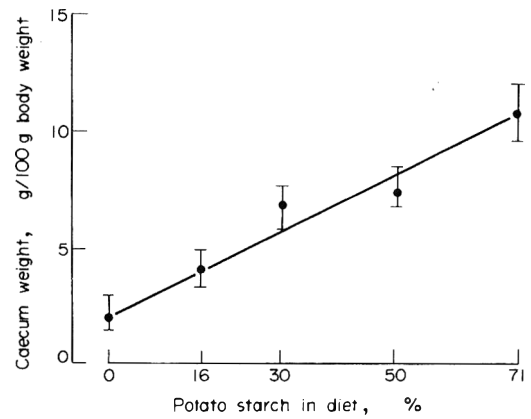

Fig. 2. Variation of the relative caecum weight with the percentage of potato starch in the diet fed to rats for 3 wk (experiment 2).

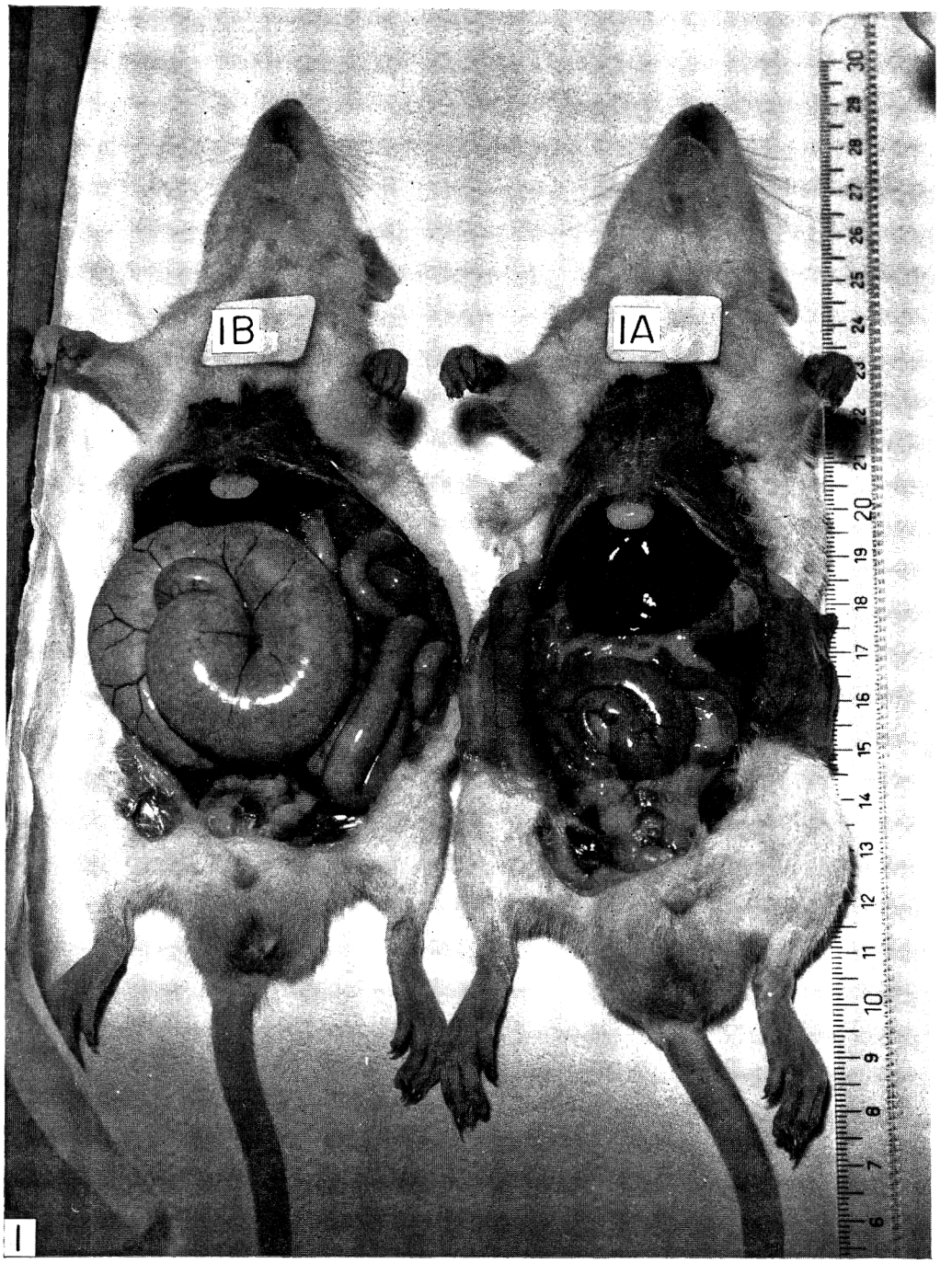

Fig. 1. Abdominal distension and caecal enlargement in a rat fed for 21 days on a diet containing 71% raw potato starch (1B) compared with a rat on 71% maize starch (1A). Rat 1B shows the compression of the liver and the distension of the rib cage associated with gross caecal enlargement.

Table 4. *Analyses of blood and caecal contents from rats fed a diet containing 71% maize starch or 71% raw potato starch for 3 wk*

Determination	No. of determinations	Values for rats fed: 71% maize starch (group 4A)	Values for rats fed: 71% raw potato starch (group 4B)
Weight of filled caecum (g/100 g body weight)	40	1·40 ± 0·07	10·33 ± 0·40***
Caecal contents			
Osmotic value (mosmol/kg)	40	236·8 ± 8·3	213·1 ± 9·1
Na^+ (μequiv./g wet weight)	40	123·0 ± 7·6	85·6 ± 4·3***
K^+ (μequiv./g wet weight)	40	35·9 ± 1·3	36·4 ± 2·3
Cl^- (μequiv./g wet weight)	40	36·9 ± 3·3	22·1 ± 1·3***
Blood†			
Na^+ (mequiv./litre)	30	119·7 ± 3·7	122·1 ± 4·7
K^+ (mequiv./litre)	30	4·42 ± 0·29	4·72 ± 0·14
Cl^- (mequiv./litre)	30	113·3 ± 2·1	115·3 ± 2·8
Urea (mg/100 ml)	30	28·7 ± 2·6	24·2 ± 4·0
GPT (mU/ml)	30	18·1 ± 1·1	28·5 ± 2·5***
AP (mU/ml)	30	75·7 ± 2·6	118·6 ± 6·0***
pH	10	7·45 ± 0·05	7·50 ± 0·06
pCO_2 (mm Hg)	10	49·5 ± 4·7	77·0 ± 3·5***
pO_2 (mm Hg)	10	62·3 ± 2·7	50·6 ± 2·0**
Lactate (mg/100 ml)	10	20·5 ± 1·3	34·9 ± 2·0***

GPT = Glutamic–pyruvic transaminase AP = Alkaline phosphatase

† Electrolyte and urea concentrations and enzyme activities were determined in serum; pH, pCO_2 and pO_2 were measured in arterial blood, and lactate concentration was determined in whole venous blood.

Values are means ± SEM for the numbers of animals stated and those marked with asterisks differ significantly (Student's *t* test) from the corresponding value for the control group (4A): ** $P < 0·01$; *** $P < 0·001$.

of the hypertrophy reported here is greater, however, than that previously described and the small and large intestines were also hypertrophied. Moreover, there was a linear dose-response relationship (Fig. 2).

Previous workers observed that ball-milling or autoclaving potato starch reduced the caecum-enlarging effect (Jelinek *et al.* 1952). In the present study it was found that autoclaving, but not ball-milling, greatly reduced the effect. The amount of physical damage to the starch granules effected by ball-milling is very variable, depending on time, ball mass, rotational velocity, moisture content and the heat generated, so that in the absence of standardized milling conditions our results and those of other workers are not easily comparable. Assessment of the degree of damage to the starch granule on the basis of reaction to pancreatic amylase (Sandstedt & Schroeder, 1960) showed that the conditions used for milling in our laboratory were not very effective and this probably accounts for the observed interlaboratory differences.

The occurrence of intact potato-starch granules in the caecal contents and faeces of test animals, correlated with the observation that raw and milled potato starches were less readily hydrolysed *in vitro* by pancreatic α-amylase than was corn starch or autoclaved potato starch (E. A. El-Harith and R. Walker, unpublished data 1974).

The extent of caecal enlargement correlated well with the resistance to hydrolysis by pancreatic amylase. On the basis of earlier evidence, it seems unlikely that a heat-labile toxic factor other than the potato starch itself is responsible for the observed caecal hypertrophy. Potato starch has a high phosphate content (Bhotiyakornkiat & Birch, 1972), which may be present in the outer layers and confer a higher degree of resistance to pancreatic amylase than maize and other cereal starches; either thermal or mechanical disruption of the granule can thus increase digestibility. Starch hydrolysate fractions which are rich in phosphate (presumably as phosphated glucose moieties) can also cause caecal enlargement in rats, probably because of the inability of endogenous intestinal enzymes to hydrolyse phosphated sugars and hence the failure of absorption of the phosphated oligosaccharides (Bhotiyakornkiat & Birch, 1972). Certain oligosaccharides, such as raffinose and stachyose present in beans, have been implicated in abnormal intestinal function and flatulence (Aspinall, Hunt & Morrison 1966; Subba Rao & Desikachar, 1964), and it may be that partially hydrolysed phosphated oligosaccharides are more significant than intact starch granules in effecting the caecal hypertrophy.

The utilization of the carbohydrate component of the diet—as assessed by its capacity to promote growth (Table 2) and by its effect on the quantity of faeces excreted (Table 3)—was clearly impaired in all animals receiving raw or milled potato starches. This impairment of utilization can be accounted for by the already mentioned resistance of potato starch to hydrolysis by pancreatic amylase.

An absence of histological abnormality from the gastro-intestinal tract has been reported previously (Leegwater *et al.* 1974) and this, together with the observation that caecal hypertrophy was almost com-

pletely reversible in 10 days after the withdrawal of potato starch from the diet (group 1B2), suggested that gastro-intestinal hypertrophy may be a process of physiological adaptation to the presence of undigested material in the diet. Leegwater *et al.* (1974) suggested that the adaptation was controlled by the osmolality of the caecal contents, and in the present work the osmolalities of caecal contents (Table 4) were comparable in test and control animals, although sodium and chloride concentrations were lower in the test group.

An alternative possibility is that there are considerable changes in the population of the intestinal microflora when large amounts of undigested fermentable material are present in the caecum, and that the microflora in some way control the caecal size. The converse situation is well-known; there are great morphological differences between the gut of germ-free and conventional rats, the former having an enlarged caecum and smaller intestinal villi, but the biochemical basis of this change is not clear (Mickelsen, 1962). Changes in the intestinal flora associated with the potato starch-induced caecal enlargement are currently being investigated and may throw further light on the problem, but a multifactorial influence seems likely. Thus, while most animals given raw potato starch in the current study had enlarged caeca with soft contents of high moisture content, occasional animals responded differently, displaying caeca which, though enlarged, contained hard solid material. In most cases the caecal microflora may have adapted qualitatively and quantitatively to deal with the increased mass of fermentable material reaching this region of the gut, with the osmotically active but unabsorbable hydrolysis products favouring fluid retention (although, as stated above, osmolality cannot be the whole explanation). In occasional cases where such microbial adaptation had not occurred, hydrolysis of the starch to osmotically active lower-molecular-weight sugars would not take place. The intact starch granules might then swell and absorb water without greatly affecting the osmotic pressure of the free water, a situation resulting in the solidified caecal contents observed. In this way, both intestinal microflora and osmotically active molecules may influence the ultimate gut morphology.

If caecal enlargement is a process of physiological adaptation, it is obviously not very efficiently controlled, in the sense that ultimately the well-being of the animal is impaired. In this study, out of 80 rats receiving raw (or milled) potato starch, at dietary levels of 30% or more, 17 died, while no deaths occurred in controls receiving maize starch. The cause of death was not immediately obvious although the physical changes associated with caecal enlargement, including compression of the liver and diaphragm, could have altered organ function (Fig. 1).

Intestinal haemorrhage and caecal obstruction were not observed in all animals that died, suggesting some other cause of death. The elevated serum alkaline-phosphatase levels (Table 4) are not unexpected in association with gross hypertrophy of the gastro-intestinal tract (Hietanen & Hänninen, 1971) but the normal histology leads to the conclusion that lesions of the gut are unlikely to have been the cause of death. Similarly although serum glutamic–pyruvic transaminase levels were almost doubled in animals showing caecal hypertrophy (Table 4) they were still within the range reported as 'normal' for rats (Street, 1970) and the histology of the liver did not reveal any pathological change likely to impair organ function.

Despite the greatly increased fluid retention in the gut of test animals, serum electrolyte levels (Table 4) and urea were normal, and kidney function and morphology appeared to be unaffected.

The most significant observations, therefore, appear to be the elevated pCO_2 and lactate levels and reduced pO_2, indicative of impaired respiratory-gas exchange. The bloated gut led to serious compression of the diaphragm and expansion of the rib cage (Fig. 1) and it appears that this condition renders adequate ventilation difficult or impossible, and results in death due to respiratory collapse.

CONCLUSIONS

On the basis of this analysis, caecal enlargement *per se* may be considered as an adaptive response, as proposed by Leegwater *et al.* (1974). In short-term studies mortality would be likely to result from secondary effects only in extreme situations. However, further work with other materials that cause caecal hypertrophy is obviously necessary to validate this conclusion and to investigate whether, over long-term tests, chronic intestinal enlargement results in abnormal function of the gastro-intestinal tract and/or other organs. Previous reports have shown that raw potato starch is poorly utilized by the chicken (D'Mello & Whittemore, 1975) and potatoes have a lower nutritive value for pigs when raw than when cooked (Whittemore, Taylor, Moffat & Scott, 1975), but effects analogous to the caecal enlargement seen in rodents were not described. Similar caecal enlargement in rodents is observed with modified starches which, like raw potato starch, are resistant to pancreatic amylase (Leegwater *et al.* 1974), and further studies in non-rodents would assist in assessing the significance of the observed gastro-intestinal effects to the use of these modified starches as stabilizers in infant foods.

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EFFETS DE L'ACIDE TANNIQUE ET DU METABISULFITE DE POTASSIUM, EN ASSOCIATION AVEC L'ETHANOL, SUR QUELQUES ACTIVITES DE DETOXICATION ENZYMATIQUE DES MICROSOMES HEPATIQUES DU RAT

D. GAILLARD et G. CHAMOISEAU

Groupe de Recherches sur la Toxicologie des Aliments et des Boissons, INSERM-U.87, Université Paul Sabatier, 2 Rue François Magendie, 31400-Toulouse, France

et

M. SUSCHETET

Station de Recherches sur la Qualité des Aliments de l'Homme, INRA, 7 Rue Sully, 2100-Dijon, France

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Résumé—Les activités de la *N*-déméthylase du pyramidon, de l'*O*-déméthylase du *p*-nitroanisole et de l'hydroxylase aromatique de l'aniline ainsi que le taux du cytochrome *P*-450, au niveau de la fraction microsomale hépatique, sont évalués chez des rats femelles soumis pendant 9 semaines à des régimes contenant de l'acide tannique, du métabisulfite de potassium et de l'éthanol administrés soit séparément soit ensemble dans toutes leurs combinaisons possibles. L'éthanol et l'acide tannique, individuellement, stimulent les activités de détoxication de quelques monooxygénases microsomales et font augmenter le taux du cytochrome *P*-450. Lorsque ces deux substances sont administrées conjointement, leurs effets deviennent additifs. Parallèlement, les concentrations en protéines et en ARN des microsomes sont augmentées en présence d'éthanol mais pas d'acide tannique. Bien que le sulfite ne semble pas avoir d'effet principal sur ces paramètres, il potentialise les effets de l'acide tannique et de l'éthanol sur le taux du cytochrome *P*-450.

Abstract—The activities of amidopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase and aniline hydroxylase, as well as the level of cytochrome *P*-450, were determined in the microsomal fraction of the livers of female rats maintained for 9 wk on diets containing tannic acid, potassium metabisulphite and ethanol either separately or together in each of the possible combinations. Given alone, ethanol and tannic acid each stimulated the detoxicating activities of the microsomal monooxygenases and increased the level of cytochrome *P*-450. When the two substances were administered together, their effects were additive. Parallel increases in the concentrations of microsomal protein and ribonucleic acid were induced by ethanol but not by tannic acid. Although the sulphite did not appear to have a significant effect on these parameters, it potentiated the effect of tannic acid and ethanol on the level of cytochrome *P*-450.

INTRODUCTION

Les microsomes hépatiques sont pourvus d'une grande diversité d'enzymes qui peuvent métaboliser une quantité innombrable de substances exogènes naturelles ou synthétisées (Conney, 1967; Gaillard et Derache, 1972). Les activités de ces enzymes microsomales peuvent être stimulées ou inhibées par de nombreuses substances (Fouts, 1970). Cependant les études concernant les interactions de plusieurs inducteurs ou inhibiteurs, administrés en même temps, sont beaucoup plus restreintes; pourtant, il est possible que leurs effets, sur les activités de détoxication enzymatique par les microsomes hépatiques, puissent se potentialiser ou exercer soit des synergismes soit des antagonismes en entraînant des modifications des actions pharmacologiques ou toxicologiques individuelles. Ainsi, il est connu, en clinique, que plusieurs médicaments administrés en même temps à des malades peuvent provoquer des accidents graves telle l'administration conjointe d'anticoagulants et de phénobarbital (Cucinell, Conney, Sansur et Burns, 1965).

Il faut noter toutefois, que très peu d'études, concernant ce sujet, portent sur les additifs alimentaires ou les substances naturelles des plantes qui se trouvent dans les aliments. Quelques études ont été entreprises concernant l'éthanol qui stimule l'activité de détoxication enzymatique microsomale, lorsqu'il est introduit dans le régime du rat (Rubin, Bacchin, Gang et Lieber, 1970; Rubin et Lieber, 1968). Lorsque l'éthanol est administré avec du phénobarbital 16–24 hr avant le sacrifice des rats, ces deux substances ont un effet additif sur l'activité des enzymes microsomales (Ariyoshi, Takabatake et Remmer, 1970). Par ailleurs, l'introduction de chlorcyclizine, de DDT et d'éthanol dans des régimes provoque individuellement des inductions de l'*O*-déméthylase du *p*-nitroanisole et fait élever le taux du cytochrome *P*-450; en combinaison, il y a une sommation de l'action de ces substances (Singlevich et Barboriak, 1971).

A la suite de ces données, nous nous sommes demandés si l'effet stimulateur de l'éthanol sur les monooxygénases microsomales ne peut pas être renforcé ou atténué par l'administration conjointe de

deux substances qui se trouvent également dans l'alimentation de l'homme: les tannins et les sulfites. En effet, les tannins sont abondants, à l'état naturel, dans des fruits (cerises, fraises, pommes), des végétaux (cacao, laitues) et également dans des boissons comme le vin, le thé et le café; les sulfites sont ajoutés en quantité non négligeable dans de nombreux produits alimentaires, entre autres le vin, le cidre, la bière, les jus de fruits, les fruits secs, les poissons séchés et la moutarde. Ces deux substances peuvent être ingérées journellement en même temps que de l'éthanol et même être associées avec lui dans certaines boissons. C'est pourquoi, il nous a paru intéressant d'étudier les effets de l'éthanol, de l'acide tannique* et du sulfite, soit séparément soit associés, sur quelques activités de détoxication enzymatique au niveau des microsomes hépatiques du rat. Ces trois substances sont administrées dans des régimes semi-synthétiques, pendant 9 semaines, dans toutes leurs combinaisons possibles. Notre expérience porte donc sur huit groupes d'animaux soumis chacun à un régime contenant soit séparément le sulfite, le tannin et l'éthanol soit les combinaisons: sulfite–tannin, sulfite–éthanol, tannin–éthanol et sulfite–tannin–éthanol. Le groupe témoin ne reçoit aucune de ces substances.

METHODES EXPERIMENTALES

Animaux et régimes. Des rats femelles SPF Wistar (provenant de l'élevage de la Station de Recherches sur la Qualité des Aliments de l'Homme, INRA, Dijon, France) âgés de 28 jours, sont divisés en huit groupes de 16 animaux chacun. Ils reçoivent comme nourriture, pendant 9 semaines, des régimes semi-synthétiques très hydratés dont la composition est indiquée dans le Tableau 1. Le sulfite (métabisulfite de potassium, E. Merck), l'acide tannique (E. Merck) et l'éthanol absolu introduits dans certains de ces régimes se substituent à une partie des glucides; le sulfite se substitue pondéralement au saccharose et l'acide tannique à l'amidon. En ce qui concerne l'éthanol, la substitution est réalisée sur une base isocalorique (30% des calories d'origine glucidique des régimes n'en contenant pas). L'aliment et l'eau de boisson sont donnés *ad lib.* La thiamine, pouvant être détruite dans les régimes contenant du sulfite, n'est pas introduite dans le mélange vitaminique mais est allouée aux animaux à la pipette deux fois par semaine.

Préparation de la fraction microsomale hépatique. Après 9 semaines de régimes, les animaux sont sacrifiés par décapitation. Le foie est prélevé immédiatement et pesé. Une partie aliquote est aussitôt plongée dans de l'azote liquide où elle est conservée jusqu'au moment des dosages. Sitôt après décongélation, le foie est broyé au moyen d'un Potter dans 4 vols de tampon phosphate de potassium (0,1 M, pH 7,4) glacé. L'azote liquide ayant modifié la texture du tissu hépatique, une première centrifugation à 26000 **g**, pendant 15 min à 4°C, permet d'éliminer les noyaux, les mitochondries et les débris cellulaires. Le surnageant est alors centrifugé à 4°C à 105000 **g** pendant 1 hr (ultracentrifugeuse MSE). Le culot, contenant les microsomes, est mis en suspension dans le tampon phosphate glacé de façon à avoir une concentration en protéines de l'ordre de 8 mg/ml.

Les concentrations en protéines et en acide ribonucléique (ARN) sont déterminées dans la fraction microsomale respectivement selon une méthode à la bromosulfophtaléine (Paul, 1961) et selon la technique de Wannemacher, Banks et Wunner (1965). Les standards de protéines sont faits avec de la sérum albumine humaine, fraction V (Nutritional Biochemicals Corp., Cleveland, Ohio); l'ARN est évalué par rapport à de l'ARN de foie de mouton (Choay, Paris).

*Les termes acide tannique et tannin sont employés indifféremment.

Tableau 1. *Composition des régimes expérimentaux administrés aux rats pendant 9 semaines*

Constituants	Composition (g) des régimes							
	Témoin	Sulfite	Tannin	Ethanol	Sulfite + tannin	Sulfite + éthanol	Tannin + éthanol	Sulfite + tannin + éthanol
Caséine	23	23	23	23	23	23	23	23
Amidon	38	38	34,8	26	34,8	26	22,8	22,8
Saccharose	28	27	28	20	27	19	20	19
Huile de maïs	5	5	5	5	5	5	5	5
Mélange salin*	4	4	4	4	4	4	4	4
Méthionine	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Agar-agar	2	2	2	2	2	2	2	2
Métabisulfite de K	—	1	—	—	1	1	—	1
Acide tannique	—	—	3,2	—	3,2	—	3,2	3,2
Ethanol	—	—	—	11,5	—	11,5	11,5	11,5
Eau	199	199	199	199	199	199	199	199
Mélange vitaminique†	1	1	1	1	1	1	1	1

*Mélange de Hubbel, Mendel et Wakeman (1937) à ces différences près (en g/kg de mélange salin): $MnSO_4.H_2O$ 3.50; $ZnCl_2$ 1,66; $CoCl_2,6H_2O$ 0,08.

†Le mélange vitaminique apporte, pour 1 kg de régime sec: vitamine A "hydrosoluble" 6000 UI, vitamine D_2 500 UI, vitamine E "hydrosoluble" 100 mg, vitamine K_1 1 mg, riboflavine 4 mg, vitamine B_6 4 mg, acide pantothénique 10 mg, amide nicotinique 50 mg, acide folique 1 mg, biotine 0,2 mg, vitamine B_{12} 0.03 mg, chlorure de choline 1000 mg, inositol 200 mg, acide *p*-aminobenzoïque 500 mg. La thiamine est administrée séparément à la pipette (environ 250 μg/jour).

La teneur en cytochrome *P*-450 est également déterminée, en employant le coefficient d'extinction molaire de 91/cm/mM (Omura et Sato, 1964).

Evaluation de l'activité de détoxication enzymatique par les microsomes hépatiques. L'activité des enzymes microsomales est estimée après incubation dans un milieu de 6 ml contenant 0,75 µmol de triphosphopyridine nucléotide, 50 µmol de glucose-6-phosphate, 0,5 UI de glucose-6-phosphate déshydrogénase, 25 µmol de chlorure de magnésium, 100 µmol de nicotinamide, 5 µmol de diphosphopyridine nucléotide réduit et 290 µmol de tampon phosphate de potassium, pH 7,4. Les concentrations en substrats sont de 5 µM pour l'aniline et le pyramidon et de 3 µM pour le *p*-nitroanisole. La quantité de protéines microsomales présente dans les 6 ml de milieu d'incubation est approximativement de 20 mg. L'incubation est menée, pendant 30 min sous agitation (150 oscillations/min), dans un appareil Gallenkamp thermostaté à 37°C, l'air servant de phase gazeuse.

Les quantités de métabolites formés à partir des trois substrats utilisés sont ensuite déterminées. L'hydroxylation aromatique de l'aniline est évaluée par l'apparition de *p*-aminophénol (Kato et Gillette, 1965), l'*O*-déméthylation du *p*-nitroanisole est estimée par la formation de *p*-nitrophénol (Kato et Gillette, 1965) et la *N*-déméthylation du pyramidon est déterminée par la formation de 4-aminoantipyrine (La Du, Gaudette, Trousof et Brodie, 1955). Les résultats sont exprimés en nmols de métabolite formé/mg de protéines microsomales en 30 min d'incubation.

Calculs statistiques. Notre expérience est du type expérience factorielle 2^3, à trois facteurs (sulfite, tannin, éthanol) chacun à deux niveaux (absence ou présence du facteur); dans les huit régimes sont représentés les trois facteurs dans toutes leurs combinaisons possibles soit séparément soit ensemble. Sur un paramètre donné, on peut dès lors évaluer l'effet propre de chaque facteur et celui qui résulte de son association avec les autres. Les variations entre les différents groupes ont été décomposées en variations dues aux effets principaux des facteurs sulfite, tannin, éthanol et aux effets d'interactions sulfite–tannin, sulfite–éthanol, tannin–éthanol et sulfite–tannin–éthanol. Les calculs ont été faits suivant la technique des polynomes orthogonaux et l'analyse de variance classique permet, grâce au test de *F*, d'exprimer la signification des résultats (Lison, 1968).

RESULTATS

Effets du sulfite, de l'acide tannique et de l'éthanol sur les poids des foies et sur les taux des protéines, de l'ARN et du cytochrome P-450 *des microsomes hépatiques du rat*

Le Tableau 2 donne les moyennes des résultats pour les paramètres poids corporel, poids des foies et taux des protéines, de l'ARN et du cytochrome *P*-450 de la fraction microsomale des animaux soumis aux huit régimes. L'éthanol ($F = 9,6$) et le tannin ($F = 104,0$) font, tous deux, diminuer le poids des animaux après 9 semaines de régime, alors qu'il n'y a pas de modification après traitement au sulfite. En ce qui concerne le poids du foie rapporté à 100 g de poids corporel, le phénomène est plus complexe; le tannin fait augmenter le poids du foie ($F = 84,4$) alors que l'éthanol le fait diminuer ($F = 5,1$). Bien que le sulfite n'ait pas d'effet principal, en combinaison avec l'éthanol ($F = 11,4$) ou le tannin ($F = 6,5$) il se produit des interactions, c'est-à-dire que les effets de ces deux substances sont modifiés en présence du sulfite qui semble atténuer leur action, ceci se retrouvant dans la combinaison sulfite–tannin–éthanol ($F = 5,3$). Cependant l'étude des effets simples montre que le tannin garde néanmoins une action prépondérante du fait que le poids des foies des rats soumis au tannin est toujours significativement plus élevé que lorsque cette substance est absente des régimes.

Seul l'éthanol provoque une augmentation des taux des protéines ($F = 9,4$) et de l'ARN ($F = 7,2$) au niveau des microsomes hépatiques. Le tannin ($F = 18,5$) et l'éthanol ($F = 12,1$) font augmenter significativement la concentration en cytochrome *P*-450 de la fraction microsomale alors que le sulfite n'a pas d'action. Lorsque les facteurs sont combinés 2 à 2, les effets ne sont qu'additifs et il n'y a pas d'interaction; par contre, lorsque les trois facteurs sont administrés dans le même régime, il y a une potentialisation du phénomène dont le sulfite serait responsable ($F = 6,2$).

Tableau 2. *Effets du sulfite, du tannin et de l'éthanol, administrés dans des régimes, sur le poids des foies et sur les taux des protéines, de l'ARN et du cytochrome* P-450 *des microsomes hépatiques du rat*

Régimes	Poids corporel (g)	Poids du foie (g/100 g poids corporel)	Protéines microsomales (mg/g de foie)	ARN microsomal (mg/g de foie)	Cytochrome *P*-450 (nmols/g de foie)
Nombre d'animaux...	16	16	14	14	14
Témoin	247,00 ± 6,26	3,18 ± 0,05	16,52 ± 0,61	2,52 ± 0,08	8,23 ± 0,74
Sulfite	250,18 ± 7,57	3,18 ± 0,04	16,50 ± 0,56	2,52 ± 0,05	6,98 ± 0,68
Tannin	202,81 ± 3,61	3,60 ± 0,05	16,77 ± 0,45	2,52 ± 0,09	8,64 ± 0,67
Ethanol	234,50 ± 5,96	3,03 ± 0,03	18,05 ± 0,64	2,69 ± 0,10	8,78 ± 0,43
Sulfite–tannin	208,93 ± 3,25	3,29 ± 0,03	18,61 ± 0,58	2,72 ± 0,07	11,61 ± 0,61
Sulfite–éthanol	228,06 ± 5,27	3,10 ± 0,03	18,57 ± 0,67	2,77 ± 0,09	10,63 ± 0,70
Tannin–éthanol	198,12 ± 2,68	3,38 ± 0,05	18,69 ± 0,61	2,77 ± 0,05	11,12 ± 0,99
Sulfite–tannin–éthanol	203,68 ± 3,80	3,44 ± 0,05	18,14 ± 0,45	2,67 ± 0,06	12,06 ± 0,79

Les valeurs représentent la moyenne ± l'erreur standard pour le nombre d'animaux de chaque groupe utilisé pour l'étude du paramètre correspondant.

Tableau 3. *Effets du sulfite, du tannin et de l'éthanol, administrés dans des régimes, sur les métabolismes de l'aniline, du pyramidon et du* p*-nitroanisole au niveau des microsomes hépatiques du rat*

		Activité enzymatique (nmols de métabolite formé/mg protéines microsomales/30 min)		
		Aniline (hydroxylation aromatique)	Pyramidon (*N*-déméthylation)	*p*-Nitroanisole (*O*-déméthylation)
Régimes	Nombre d'animaux...	7	7	7
Témoin		12,16 ± 0,59	9.01 ± 0,51	19,44 ± 1,06
Sulfite		12,26 ± 1,02	8.68 ± 0,64	19,14 ± 1,07
Tannin		12,36 ± 0.30	7,68 ± 0,31	23,15 ± 1.05
Ethanol		18,05 ± 0,64	7,92 ± 0,38	25,43 ± 1,37
Sulfite–tannin		12,02 ± 0,99	8.08 ± 0,51	28,81 ± 1,72
Sulfite–éthanol		20,61 ± 1,12	8,52 ± 0,51	28,34 ± 1,46
Tannin–éthanol		18,42 ± 0,69	7,36 ± 0,31	28,64 ± 1,36
Sulfite–tannin–éthanol		17.46 ± 0.73	7,71 ± 0,34	32,54 ± 0,42

Les valeurs représentent la moyenne ± l'erreur standard pour les sept animaux de chaque groupe utilisés pour l'étude enzymatique correspondante.

Effets du sulfite, de l'acide tannique et de l'éthanol sur l'activité de détoxication des microsomes hépatiques

Les résultats rapportés dans le Tableau 3 montrent que l'éthanol provoque des augmentations significatives des activités des enzymes, au niveau des microsomes hépatiques, en présence de deux substrats utilisés lors de notre expérience: l'aniline ($F = 100,5$) et le *p*-nitroanisole ($F = 69,2$). Avec le tannin, il y a augmentation de l'activité de l'*O*-déméthylase du *p*-nitroanisole ($F = 20,0$) et une légère modification de l'activité de la *N*-déméthylase du pyramidon ($F = 5,8$). Ces effets stimulateurs des activités de détoxication enzymatique se retrouvent dans la combinaison des facteurs, mais cependant ils ne sont qu'additifs et il n'y a pas d'interaction.

DISCUSSION

En ce qui concerne l'effet individuel de l'éthanol, après 9 semaines d'administration dans des régimes, nos résultats recoupent ceux donnés dans la littérature bien qu'ils ne nous permettent pas de savoir s'il s'agit d'une induction enzymatique (Lieber, 1973) ou d'une stimulation des monooxygénases microsomales (Ioannides et Parke, 1973). En effet, dans nos conditions expérimentales, l'éthanol fait augmenter les vitesses de métabolisme de l'aniline et du *p*-nitroanisole; nos résultats montrent par ailleurs que le traitement à l'éthanol n'aurait pas d'effet significatif sur la *N*-déméthylase du pyramidon. L'activation des enzymes de détoxication s'accompagne, dans nos conditions expérimentales, d'augmentations des taux de l'ARN et des protéines au niveau des microsomes comme cela se produit classiquement lors de ce phénomène. Il y a également une élévation importante de la concentration en cytochrome *P*-450 recoupant, là encore, les résultats trouvés dans la bibliographie.

Lors d'un travail antérieur, nous avions mis en évidence une inhibition de l'activité de détoxication enzymatique des microsomes après injection intrapéritonéale d'acide tannique, alors qu'il n'y avait aucun effet lors d'administration orale par intubation, assurément dû au fait que le tannin ne traverse pas, dans ces conditions, la barrière intestinale en quantité suffisante pour produire le même phénomène (Gaillard, Mitjavila et Derache, 1974). Par contre, après administration dans les régimes à dose nettement moins importante, nos résultats montrent qu'il y a stimulation de l'*O*-déméthylase du *p*-nitroanisole et, dans une moindre mesure, de la *N*-déméthylase du pyramidon. Il n'y a pas parallèlement de modifications des taux des protéines et de l'ARN; il est possible qu'après passage dans les cellules hépatiques, le tannin se trouve lié dans les noyaux à l'ADN, interférant ainsi dans la transcription ADN–ARN (Racela, Grady et Svoboda, 1967). Lorsque l'acide tannique et l'éthanol sont administrés ensemble il y a une sommation des activations de l'*O*-déméthylase du *p*-nitroanisole; des faits similaires ont été signalés lors de combinaison, dans des régimes, d'éthanol et de deux inducteurs: le DDT et la chlorcyclizine (Singlevich et Barboriak, 1971). Ce phénomène se retrouve pour le cytochrome *P*-450 où l'augmentation de sa concentration est additive en présence d'éthanol et d'acide tannique.

Le sulfite n'a pratiquement pas d'influence sur la détoxication enzymatique dans nos conditions expérimentales; il est possible que le métabisulfite de potassium utilisé dans les régimes soit détruit au pH de l'estomac. Néanmoins lorsqu'il est administré conjointement à l'éthanol et à l'acide tannique, dans le même régime, il y a une potentialisation de l'augmentation du taux du cytochrome *P*-450, c'est-à-dire que l'élévation de la concentration en cytochrome *P*-450 est supérieure à celle qu'on devrait normalement observer en additionnant les effets principaux des trois substances. Ceci n'est pas à négliger du fait que le cytochrome *P*-450 joue un rôle important dans les phénomènes de détoxication enzymatique; en effet, il est responsable de l'activation de l'oxygène moléculaire (Omura, Sato, Cooper, Rosenthal et Estabrook, 1965) et de la liaison avec le substrat (Imai et Sato, 1966: Schenkman, Remmer et Estabrook, 1967), per-

mettant ainsi l'oxydation de nombreuses substances exogènes (Gillette, 1966).

Notre expérience ne permet pas de définir les mécanismes par lesquels se produisent ces phénomènes. Cependant, du fait de la consommation importante des boissons alcooliques qui d'ailleurs peuvent contenir des tannins et des sulfites, composés se trouvant en quantité relativement importante dans l'alimentation de l'homme, le problème concernant la stimulation des monooxygénases microsomales par ces trois substances n'est pas à négliger.

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ACUTE AND SHORT-TERM TOXICITY OF OCHRATOXIN A IN 10-DAY-OLD CHICKS

P. GALTIER, J. MORE and M. ALVINERIE

Station de Pharmacologie-Toxicologie INRA, 180 Chemin de Tournefeuille, 31300 Toulouse, France

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Abstract—Ten-day-old chicks were given ochratoxin A orally, either in a single dose or by daily administration over 10 days. The primary effects of both forms of treatment were prostration, cachexy and growth retardation. The LD_{50} values found were 10·67 mg/kg for single doses and a total of 13·97 mg/kg for multiple doses. Post-mortem examination revealed pale livers and kidneys, together with hypertrophy of the gall-bladder and intestinal congestion. Fatty change was the principal histological finding in both livers and kidneys, with some necrosis of the hepatocytes and the presence of eosinophilic hyaline casts in the kidney tubules. The similarity of the two LD_{50} values suggested the possibility of accumulation of the toxin in the chick.

INTRODUCTION

Ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin linked through the 7-carboxyl group by an amide bond to L-phenylalanine) is produced by *Aspergillus ochraceus* Wilh. (van der Merwe, Steyn & Fourie, 1965) and *Penicillium viridicatum* (van Walbeek, Scott, Harwig & Lawrence, 1969). It has been found as a natural contaminant in corn in the USA (Shotwell, Hesseltine & Goulden, 1969) and France (P. Galtier, unpublished data 1972), in barley in Denmark (Krogh, Hald & Pedersen, 1973) and in feedstuffs in Canada (Scott, van Walbeck, Kennedy & Anyeti, 1972).

The results of acute tests (Galtier, More & Bodin, 1974) and of short-term studies (Galtier, Bodin & More, 1975) in rats indicated some accumulation of toxin in the animal. The investigation described here was a similar investigation carried out in 10-day-old chicks and involving oral administration of either a single dose or of ten daily doses. Prior tests had been carried out only in day-old chicks or in broiler chickens and no comparison had been made between acute and more prolonged treatment (Chu & Chang, 1971; Huff, Wyatt, Tucker & Hamilton, 1974; Peckham, Doupnik & Jones, 1971).

EXPERIMENTAL

Animals and diet. Leghorn chicks, raised in the laboratory from one batch and with a mean body weight of 86·5 ± 1·6 g on day 10 after hatching, were divided randomly into groups of seven or eight. Each group was housed in a separate wire cage (surrounding temperature 25–27°C) and the birds were given Magda starter first age feed (Ets Vialars, Toulouse) and water *ad lib.*

Experimental procedures

Dosage. Ochratoxin A was administered by oesophageal intubation in an isotonic solution of sodium bicarbonate (14 g/litre). The dosage volume in all cases was 0·4 ml/80 g body weight (5 ml/kg) and this volume of vehicle alone was administered to a control group. Acute toxicity was determined by dosing groups of seven or eight chicks on day 10 after hatching with a single dose from a logarithmically equidistant series ranging from 4 to 16 ml/kg (as solutions containing 0·8–3·2 mg toxin/ml). In the short-term test, the doses ranged from 1 to 8 mg/kg/day (in concentrations of 0·2–1·6 mg/ml) and were given each morning from day 5 to 14 after hatching.

Body weights. Each chick was weighed each morning for 10 days after the single dose or, in the short-term study, throughout treatment and then on every second or third day for 3 wk. A factorial analysis was performed on the results obtained in the period immediately following the single administration, in the period of short-term treatment and in the period after this treatment. A Newman–Keuls test facilitated a comparison of average values and thus the determination of significant differences in each period.

Calculation of lethal doses. The acute and 10-day LD_{50} values were calculated by the probit method. In the case of the 10-day study, the LD_{50} was calculated as the total cumulative dose causing the death of 50% of the birds. It was subsequently possible to calculate a coeffecient of detoxication (D), equal to the ratio of the 10-day LD_{50} to the acute LD_{50}. This type of comparison was recommended by Beauvillain (1952).

Histology. Tissue sections were stained with haemalun–eosin for morphological examination and with periodic acid–Schiff (PAS) reagent to demonstrate hepatic glycogen (Gabe, 1968). One-third of the birds treated were examined histologically, sections being prepared from the liver, kidney, colon, adrenal gland and gall bladder from each chick.

RESULTS

General observations

Both the single and multiple doses had similar effects, the most significant of which were a reduction in spontaneous activity, huddling, hypothermia and

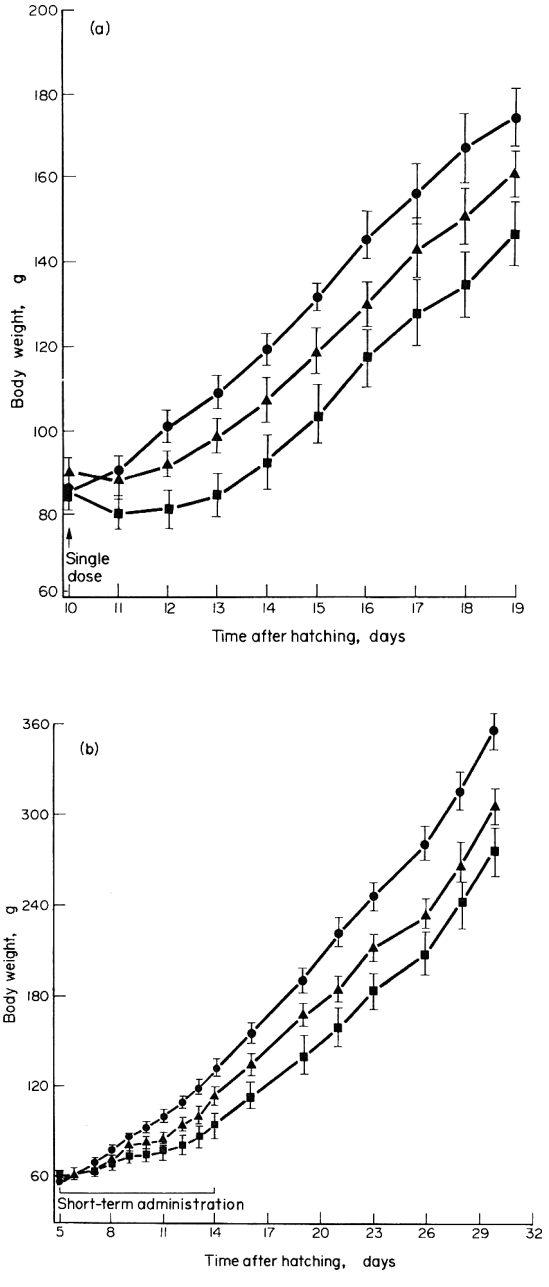

Fig. 1. Mean body weights (± SEM) of chicks in the control groups (●) and in the groups treated (a) with a single dose of 4 (▲) or 8 (■) mg ochratoxin A/kg or (b) with doses of 1 (▲) or 2 (■) mg/kg/day for a total of 10 days.

cachexy. These effects were dose-related. On the other hand, the chicks retained their reflexes and sensitivity to pain. Other signs frequently observed were tremors, eyelid ptosis and diarrhoea.

Body-weight changes

The mean body weights of chicks given a single dose of 4 or 8 mg ochratoxin A/kg or repeated doses of 1 or 2 mg/kg/day are compared with those of the corresponding control groups in Fig. 1. Factorial analysis and the Newman-Keuls test revealed highly significant ($P < 0.01$) differences between the body-weight curves of the treated chicks and those of the controls following either type of treatment.

Survival

The survival period after the acute dosage was dose-related but in all cases exceeded 48 hr. Table 1 shows the period of survival (in relation to the number of daily doses) after administration of 4 mg/kg/day.

Lethal doses

Table 1 also shows the percentage of deaths occurring and the acute and short-term median lethal doses, together with their standard errors, after calculation by the probit method. The ratio of these two parameters, or coefficient of detoxication (D), can thus be calculated as 1·31.

Post-mortem examination

Chicks that died during the test showed no significant changes externally, but their livers were pale and there was hypertrophy of the gall bladder. The kidneys were also paler than those of the control birds but had very clear vascular arborescent markings. In some cases, hyperaemia was apparent in the gastro-intestinal tract, particularly in the colon and duodenum.

Histological examination

More than 80% of liver sections revealed large areas of fatty change and, in addition, necrotic zones affected approximately one-third of the birds examined (Fig. 2). Inflammatory cells were also observed

Table 1. *Deaths induced in chicks by ochratoxin A given in single doses of 4·0–16·0 mg/kg or in daily doses of 4 mg/kg/day*

No. of doses given	Total dose administered (mg/kg)	Deaths (%)*
Single-dose study		
1	4·00	0
	5·66	0
	6·74	12
	8·00	12
	9·51	37
	11·32	62
	13·46	75
	16·00	87
Multiple-dose study		
1	4·0	0
2	8·0	11
3	12·0	33
4	16·0	66
5	20·0	77
6	24·0	100

*In groups of seven or eight chicks. The median lethal doses derived from these figures by the probit method were a single dose of 10·67 mg/kg (standard error 9·43–11·45 mg/kg) in the acute study and a cumulative dose of 13·97 mg/kg (12·63–15·47 mg/kg) for the multiple-dose treatment.

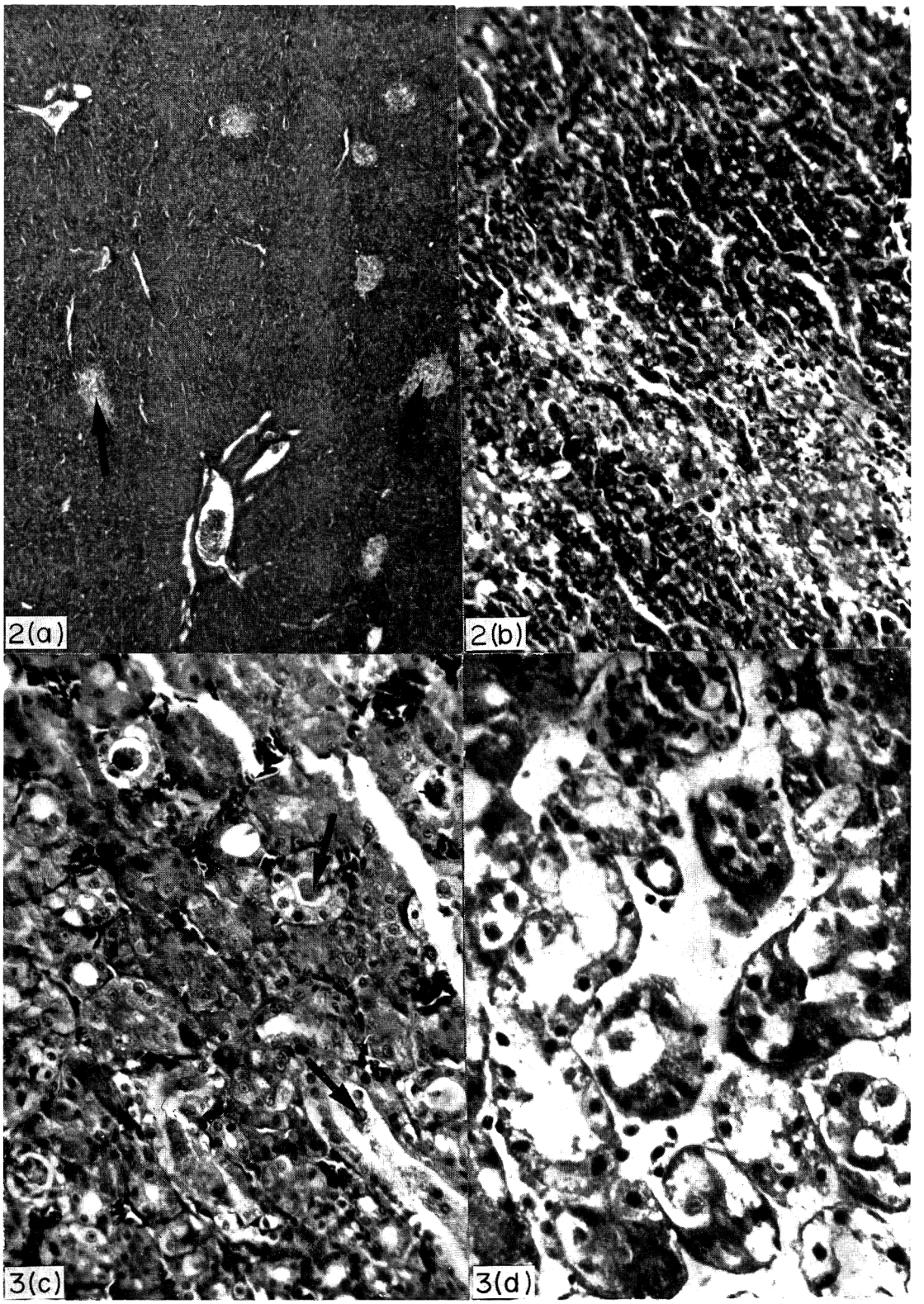

Fig. 2. Effects of ochratoxin A on the liver of the chick, showing (a) pale necrotic areas (arrowed) in the parenchyma and (b) a pale necrotic area with inflammatory cells and dark hepatocytes filled with pale fatty droplets. Haemalum-eosin × 50 (a) and × 100 (b).

Fig. 3. Effects of ochratoxin A on the chick kidney, showing (c) eosinophilic hyaline casts in the tubules (arrowed) and (d) tubular separation and vacuolation and nuclear pyknosis of the epithelial cells. Haemalum-eosin × 240 (c) and × 400 (d).

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occasionally. Finally, hepatic haemorrhages were apparent in 16% of the treated birds.

In all of the birds examined, the epithelium of the kidney tubules showed fatty degeneration, while eosinophilic hyaline casts were found in both the convoluted and collecting tubules in more than 80% (Fig. 3). Finally, microhaemorrhages of both the cortex and medulla were discovered in one-third of the birds examined.

No lesions were detected in the colon and adrenal glands. In the hypertrophied gall bladders, there was a perceptible flattening of the surface epithilium, without any change in the underlying structures.

DISCUSSION

As in the rat studies, ochratoxin A reduced bodyweight gain in chicks given single or multiple doses. Weights of the treated animals were still significantly lower than the control value 20 days after completion of the 10-day treatment, demonstrating the persistence of the toxic effect, and possibly of the toxin itself, within the organism. The latter inference was supported by the calculation of the coefficient of detoxication (D) from the two LD_{50} values (Beauvillain, 1952).

A value of D (the ratio of the multiple to the acute LD_{50}) around 1 suggests the possibility of accumulation, but if D is greater than 3, a significant elimination mechanism, which would permit the long-term administration of a drug, is indicated. The D value of 1·31 determined in this study indicates that ochratoxin A is only slowly metabolized and therefore accumulates in the chick as in the rat (Galtier *et al.* 1975). This conclusion underlines the need for a study of the fate of this toxin in the bird to determine whether, as in the rat, the mycotoxin (or perhaps one of its metabolites) binds with the plasma proteins and, in particular, with the albumin (Galtier, 1974).

The median lethal doses were greater than those previously determined in day-old chicks and broiler chickens by Peckham *et al.* (1971) and Huff *et al.* (1973), respectively. These differences may have been due to a lower sensitivity in the birds used in this test. A diminution of sensitivity has been observed in older birds, and a previous test on day-old chicks of the same strain produced an LD_{50} value of 5·4 mg/kg. These findings correspond to those of Huff *et al.* (1973) in respect of toxicological parameters in broilers.

The hepatotoxic and nephrotoxic effects observed in this study have been documented in the chick studies of Peckham *et al.* (1971) and Huff *et al.* (1973). The aetiology of these pathological changes may lie in the inhibition of mitochondrial respiration (Meisner & Chan, 1974; Moore & Truelove, 1970), but it remains to be determined why the nephrotoxic effect is the principal characteristic of ochratoxicosis in all species from chicks to pigs.

As in the rat study, numerous microhaemorrhages were discovered. It may be that ochratoxin A affects haemostasis by inhibiting the synthesis of vitamin K-dependent factors, in view of the coumarin-like structure of the toxin. It is possible also that one feature of its effect on the liver is a profound reduction in the synthesis of proteins and especially of plasma factors involved in haemostasis. Finally, ochratoxin A may have a toxic effect on the bone marrow, affecting particularly the formation of platelets. Relevant in this context is the study of Doerr, Huff, Tung, Wyatt & Hamilton (1974), in which ochratoxin A caused significant increases in recalcification and prothrombin times but not in the clotting time of whole blood.

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SHORT PAPERS

NULL CARCINOGENIC EFFECT OF LARGE DOSES OF NITROSOPROLINE AND NITROSOHYDROXYPROLINE IN WISTAR RATS

J. E. NIXON, J. H. WALES, R. A. SCANLAN, D. D. BILLS and R. O. SINNHUBER

Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331, USA

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Summary—The nitrosamino acids, nitrosoproline or nitrosohydroxyproline, were given orally to weanling rats in four weekly doses totalling 290 mg/animal and the rats were observed for their lifetime. Negative controls received water and positive controls were given diethylnitrosamine. The rats were maintained on a basal semi-purified diet or a basal diet containing cyclopropenoid fatty acids. No tumorigenic effect was seen in rats given nitrosoproline or nitrosohydroxyproline. Diethylnitrosamine produced a high incidence of hepatomas, as expected. Cyclopropenoid fatty acids had no significant effect on the carcinogenicity of the nitrosamino acids.

Introduction

N-Nitroso compounds formed in food or in the stomach from nitrite and amines may be involved in human cancer (Lijinsky & Epstein, 1970; Sebranek & Cassens, 1973; Sen, Smith & Schwinghamer, 1969). Nitrosation of secondary amines with nitrite has been demonstrated *in vitro* (Mirvish, 1971; Mirvish, Sams, Fan & Tannenbaum, 1973; Sen *et al.* 1969) and *in vivo* (Lijinsky & Greenblatt, 1972; Mirvish & Chu, 1973; Sander & Schweinsberg, 1972; Sander, Schweinsberg & Menz, 1968). Lung tumours were observed in mice fed nitrite and the secondary amines, piperazine, morpholine and *N*-methylaniline (Greenblatt, Mirvish & So, 1971). However, Greenblatt & Lijinsky (1972) failed to produce lung tumours in mice by concurrent, prolonged administration of sodium nitrite with the secondary amines, proline, hydroxyproline and arginine, and concluded that nitrosoproline (NOPro), the nitrosation product of proline, was non-carcinogenic in their mouse assay system. Greenblatt, Kommineni & Lijinsky (1973) fed sodium nitrite together with the amino acids, proline, hydroxyproline and arginine, to MRC rats for their lifetime and observed no tumorigenic effect from the dietary combination of nitrite and secondary amine. Garcia & Lijinsky (1973) then fed low levels of the nitrosamino acids, NOPro and nitrosohydroxyproline (NOHyPro), to rats for 75 wk and found no carcinogenic activity.

Previous studies to determine the carcinogenic implications of nitrosation of the secondary amines, proline and hydroxyproline, involved long-term feeding trials. We decided to investigate the carcinogenic activity of NOPro and NOHyPro in rats given large doses over a short period and then observed for their lifetime. Cyclopropenoid fatty acids (CPFA), which act as co-carcinogens in trout (Sinnhuber, Lee, Wales & Ayres, 1968), were fed to some rats in the hope of increasing the sensitivity of the biossay.

Experimental

Each experimental group consisted of 26 male weanling Wistar rats from a closed Oregon State University colony. The rats were given water and a semi-purified diet, containing 23·3% casein, 65·2% glucose, 4·3% salt mix, 2·2% vitamin mix and 5% corn oil, *ad lib.* When CPFA was fed at 1000 ppm in the diet, 0·2% *Sterculia foetida* oil was added in the corn oil. Pairs of rats were bedded on ground corn cobs in 13 × 10 × 8 in. polycarbonate cages equipped with an air-filter barrier.

NOPro and NOHyPro were synthesized in our laboratory by the procedure of Lijinsky, Keefer & Loo (1970). Diethylnitrosamine (DEN), used as a positive control, was purchased from Eastman Kodak Co., Rochester, N.Y., and redistilled before use. The test compounds, in aqueous solution, or equivalent quantities of water were administered by stomach tube once weekly for 4 wk at the beginning of the experiment. The dose schedule for each group is shown in Table 1.

All rats were weighed weekly to measure initial growth and to monitor general health. Rats were autopsied when they died or became moribund. Organ weights were recorded and tissue from liver, kidney and lung was fixed in Bouin's solution. Sections, 4 μm thick, were stained with haematoxylin and eosin for histological examination.

The significance of the effects of the various treatments on survival time and on body and organ weights was assessed using Student's *t* test.

Results

Growth, survival and weight

Except for the positive control group given DEN, there was no significant difference in growth curves, survival time or organ-weight data between rats

Table 1. *Experimental design and dose regimen for studying the effect of large doses of nitrosamino acids in rats*

Group*	Treatment	Concn of nitroso compound† (mg/ml)	Total dose administered (mg)
1	Water (control)	0	0
2	DEN	10	60
3	NOPro	50	290
4	NOHyPro	50	290
5	NOPro + CPFA	50	290
6	NOHyPro + CPFA	50	290
7	CPFA	0	0

DEN = Diethylnitrosamine NOPro = Nitrosoproline
NOHyPro = Nitrosohydroxyproline CPFA = Cyclopropenoid fatty acids

* Each group consisted of 26 male rats.
† Group 2 received 1·0, 1·3, 1·7 and 2·0 ml in wk 1, 2, 3 and 4, respectively, while other groups received 1·0, 1·2, 1·6 and 2·0 ml in consecutive weeks, the increases in the volume of consecutive doses being adjusted to maintain the same approximate dose in mg/kg body weight.

receiving the basic diet and those receiving combinations of NOPro, NOHyPro and CPFA. The rats given DEN exhibited acute toxicity and five died within 3 months of treatment. Surviving rats in this group grew at rates similar to those seen in the other groups until the onset of hepatoma development. The mean survival time (210 days) for rats given DEN was significantly less ($P < 0·01$) than for the other groups (Table 2) and the development of hepatomas in these rats caused their mean liver weight to be significantly higher ($P < 0·01$) than that of the other rats, while their body and kidney weights were significantly lower ($P < 0·05$). Spleen, heart and lung weights were similar in all the rats. Survival time was variable in all the groups and most deaths were from respiratory ailments. However, apart from the group given DEN, survival was good, with 30% of the animals living beyond 2 yr.

Tumour incidence

The tumour incidence is shown in Table 2. A high incidence of hepatomas occurred in the positive-control rats, as expected. Six lungs from that group had hepatic metastases and four rats had kidney adenomas. The only other group with confirmed neoplasia was group 5 (NOPro + CPFA) in which one rat had a hepatoma, a kidney adenoma and possibly a spleen adenoma. Two rats in the control group (1) had tumours, one a mammary tumour and one a skin fibroma on the jaw, but these tumours and the possible spleen adenoma in the rat in group 5 were not examined microscopically. Very few miscellaneous, spontaneous tumours were observed in any group.

Histopathology

The liver tumours were all hepatocellular carcinomas. Those induced by DEN (group 2) contained many degenerating cells with yellow pigment. Within many of the tumours were areas of pseudo-bile ducts, sub-spherical rather than tubular in shape. In a few instances, large tumours were almost entirely composed of pseudo-bile ducts. Some tumours had many spherical vacuoles presumed to have contained lipid. The nuclei of the hepatoma cells were usually much enlarged and vesiculated. A few tumours had become divided by strands of connective tissue and the islets so formed were assuming a ductal configuration. The one rat in group 5 with a hepatoma displayed a variety of neoplastic and preneoplastic nodules together with "cystic lesions" (Stewart & Snell, 1957) varying widely in size.

All the kidney tumours resembled the tubule adenomas found by Lee, Wales & Sinnhuber (1969) in rats fed aflatoxin B_1.

Table 2. *Incidence of tumours in rats treated with large doses of nitrosamino acids over a short period*

Group	Treatment	Mean survival time* (days)	Number examined histologically	No. with tumours			
				Liver	Lung	Kidney	Other†
1	Control	548 ± 240	24	0	0	0	2
2	DEN	210 ± 110	18	15	6	4	0
3	NOPro	615 ± 240	23	0	0	0	0
4	NOHyPro	607 ± 240	24	0	0	0	0
5	NOPro + CPFA	600 ± 210	24	1	0	1	1
6	NOHyPro + CPFA	506 ± 250	19	0	0	0	0
7	CPFA	579 ± 200	26	0	0	0	0

DEN = Diethylnitrosamine NOPro = Nitrosoproline NOHyPro = Nitrosohydroxyproline
CPFA = Cyclopropenoid fatty acids

*Mean ± standard deviation.
†Tumours not confirmed by histological examination.

The lung tumours in group 2 (DEN-treated) were hepatic metastases. These tumours were small but numerous. In one case several clusters of neoplastic cells were found in arterial sections.

Discussion

Induction of neoplasms with nitrite and some secondary amines has been demonstrated (Sander *et al.*, 1968; Sander & Schweinsberg, 1972), but induction of tumours with the nitrosamino acids, NOPro and NOHyPro, has not. Greenblatt *et al.* (1973) suggested that failure of concurrent long-term feeding of nitrite with proline, hydroxyproline or arginine to produce tumours was due to insufficient decarboxylation of the nitrosamino acids formed in the stomach. In the study now reported, four large oral doses of one of two nitrosamino acids, given weekly over a 3-wk period to weanling rats, induced no tumours during the lifetime of the rats. Each dose was approximately 650 mg/kg body weight and ranged from 50 to 100 mg/rat. That dose was large compared with approximately 25 mg nitrosamino acid/kg body weight administered daily in the long-term study (Garcia & Lijinsky, 1973). Thus relatively large amounts of substrate would have been subjected to decarboxylase activity following intubation, possibly to yield an effectively carcinogenic level of *N*-nitrosopyrrolidine or *N*-nitrosohydroxypyrrolidine. However, since *N*-nitrosopyrrolidine, the decarboxylation product of NOPro, is carcinogenic in rats (Greenblatt *et al.* 1973), decarboxylation apparently did not occur under the conditions of this study. Either the decarboxylase is not functional or NOPro or NOHyPro are not substrates. This study supports the reports of Greenblatt *et al.* (1973) and Garcia & Lijinsky (1973) that the nitrosamino acids are probably not carcinogenic and do not undergo decarboxylation in the stomach, extending their chronic findings to include high-dose, short-term exposure to NOPro and NOHyPro.

The addition of CPFA to the basal diet of rats exposed to NOPro and NOHyPro had no significant effect on the induction of tumours. Although one rat treated with CPFA and NOPro did develop tumours, the small number of rats did not allow definite conclusions to be drawn. The lack of effect was not surprising, however, since CPFA may exert a co-carcinogenic effect and the nitrosamino acids did not show carcinogenic activity.

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TOXIN PRODUCTION BY 50 STRAINS OF PENICILLIUM USED IN THE CHEESE INDUSTRY

P. LAFONT and J. LAFONT

Unité de toxicologie alimentaire de l'I.N.S.E.R.M., 44 rue du Chemin de Ronde, Le Vésinet, 78110,

J. PAYEN

Laboratoire de microbiologie, E.N.S.A.I.A., 20 rue Sainte Catherine, 54000 Nancy,

and

E. CHANY, G. BERTIN and C. FRAYSSINET

Institut de Recherches Scientifiques sur le Cancer, 7 rue Guy Mocquet, B.P. no. 8, 94800 Villejuif, France

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Summary—The production of toxins by 50 strains of Penicillium (including 29 of *P. roqueforti* and two of *P. camemberti*) isolated from cheeses and cultivated on Czapek's medium was investigated in three different tests, involving the determination of acute toxicity in the ip-treated mouse and in the chick embryo and inhibition of the growth of rat-hepatoma (LF) cells *in vitro*. About half of the extracts showed no toxicity, but extracts of two strains caused the rapid death of injected mice, nine gave a clear-cut positive response in two tests and six gave a strongly positive response in only one of the three tests. The varied responses obtained with the three tests clearly demonstrated that the toxic effects produced could not be related to a single toxin. The PR toxin of Wei *et al.* (*Appl. Microbiol.* 1973, **25**, 111) was not found in the most toxic extracts. It is not possible to extrapolate from these experimental results. They provide evidence of the toxigenic potential of the strains studied, but further studies are required to determine whether the toxins produced by these strains of Penicillium on a synthetic medium play a role in human public health.

Introduction

The question of the possible production of toxins by Penicillia used in the manufacture of cheeses has been raised following a relatively recent series of publications. Tsubaki (1956) imputed some fatal intoxications seen in cattle to the development of *Penicillium roqueforti* on ensilaged grains and, more recently, Still, Wei, Smalley & Strong (1972) isolated *P. roqueforti* from grain and silage responsible for abortions in cows. Chloroform extracts of this mould were found to be toxic to the rat. Wei, Still, Smalley, Schnoes & Strong (1973) suggested that this toxicity was linked to the presence of PR toxin, a substance that these authors isolated from extracts of cultures of *P. roqueforti* strain NRRL 849. The structure of PR toxin was established by Wei, Schnoes, Hart & Strong (1975). However, from a strain of *P. roqueforti* isolated from rice, Kanota (1968) separated three substances with toxic activities apparently different from those of PR toxin.

In a study of Penicillium strains used in the cheese industry, Still *et al.* (1972) observed that a strain used in the maturation of Roquefort cheese did not produce a toxin active in the rat. In contrast, Gibel, Wegner & Wildner (1971) produced evidence of a carcinogenic action by *Penicillium camemberti* var. *candidum* in Wistar rats after oral administration or sc injection. More recently, P. Scott (personal communication 1975) succeeded in isolating Roquefortine from *P. roqueforti* mycelium. This is an alkaloid with neurotoxic properties and has caused death when injected ip into mice.

In the work reported here, we have tried to determine more systematically whether it is possible to detect the production of toxins by strains of Penicillium used in the maturation of blue-moulded or soft-centred cheeses.

Experimental

Strains. Unless otherwise indicated (in Table 1) the strains studied were isolated from a variety of French, German, Italian and British fermented cheeses with a soft pulp and an outer crust, which was also fermented in some cases. The strains were isolated on malt-extract agar plates and were grouped according to series: *P. roqueforti* (PR), *P. camemberti* (PC) and others (P).

Extraction. Cultures of these micromycetes were grown in modified liquid Czapek's medium (Brian, Dawkins, Grove, Heming, Love & Norris, 1961) after inoculation by a method described elsewhere (Lafont & Lafont, 1974). The cultures were incubated at 25°C for 9 days. The mycelium and medium were extracted twice with acetone-water (80:20, v/v) at room temperature in the dark. After evaporation of the acetone, the aqueous phase was extracted with chloroform. The chloroform solution was evaporated and the residue was washed with hexane and dissolved in ethanol.

Table 1. *Results of the biological screening of extracts of Penicillium cultures by testing in chick embryos, cell cultures and mice*

No.	Species	Chick embryos*	Cell cultures†	Mice‡
390	PR	<20	+++	+
424	PR	150	+++	++
431	PR	<20	++	0
432	PR	<20	+	+
457–1	PR	<25	–	–
457–2	PR	<20	–	–
457–3	PR	<10	–	–
457–4	PR	<20	+	+
457–5	PR	<20	+	++
457–6	PR	<10	+	0
520–1	P	<20	++	0
520–5	P	<20	++	++++
520–7	P	<20	+	+
528–1	P	<10	+	0
528–2	P	<10	+	+
528–3	P	<10	+++	+
528–4	P	<10	++	++
528–5	P	<10	+	0
528–6	P	<10	+	+
529–1	P	<10	0	++
529–2	P	<10	+	++
530–1	P	<10	0	+
530–2	P	<10	+	+
530–3	P	<20	+	+
549	PR	<20	++	+
551–0	PR	<25	++	+
551–1	PR	<25	+	++
551–2	PR	50	+	+
551–3	PR	<20	++	+
551–4	PR	<20	0	0
551–5	PR	200	++++	+
551–6	PR	<20	+	+
551–7	PR	500–600	+++	+
552–0	PR	<20	+	+++
553–1	PR	<20	+++	++++
553–2	PR	<20	+++	0
554§	PR	<20	+++	++
564–0	PC	<20	+	++
565–0	PC	<20	0	+
567–2	PR	100	+++	+
632–1	PR	800–1100	+++	++
632–2	P	400–500	++++	++
632–3	PR	800–1250	++++	+
633	P	80	++++	+
634	P	300–400	++++	+++
635‖	P	100	ND	ND
636‖	PR	150	ND	ND
637	P	100	ND	ND
638	PR	200	ND	ND
639	PR	100	ND	ND

ND = Not determined

*Expressed as no. of LD_{50}s/g dry culture.

†Scale: ++++, toxic; +++, ++ and +, inhibiting by 75, 50 and 25%, respectively; 0, inactive.

‡Scale: ++++, nearly 100% mortality following an ip dose equivalent to 7·5 g dry culture/kg; 0, 100% survival following an ip dose equivalent to 25 g dry culture/kg; +++, ++ and +, graded intermediate effects.

§Strain isolated from a sample of Egyptian soil.

‖Strain isolated from mouldy bread.

An aliquot of the extract was analysed by thin-layer chromatography on Brinkman silica gel using chloroform-methanol (94:4, v/v) as the developing solvent, and the presence of fluorescent compounds and PR toxin was determined by ultraviolet irradiation.

Biological tests

The toxicity of the extracts was determined by three tests, namely inhibition of growth of the chick embryo, inhibition of multiplication of cell cultures and acute toxicity in the mouse.

Chick-embryo test. Fertile eggs from Leghorn hens were inoculated according to the method of Lafont & Frayssinet (1969). Each dilution of extract was inoculated into 20 eggs.

Cell cultures. Since a preliminary study of the comparative sensitivities of cultures of normal liver hepatocytes and of LF cells derived from an experimental rat hepatoma had shown that the two types responded in an identical manner, this work was carried out on LF cells for practical reasons. These are fast-growing cells that have retained some of the metabolic potentialities of the original hepatocytes. They were seeded at a concentration of 100,000 cells/ml in MEM (minimum Eagle medium) containing 10% calf serum. The test extracts were added at the beginning of the culture period in various concentrations in dimethylsulphoxide (DMSO), the volume of DMSO used being 20 μl/3 ml medium. The number of cells obtained was determinated with a Coulter counter 24 and 48 hr after seeding, each sample being tested in triplicate. Five dilutions of each extract were tested in series to demonstrate either toxicity, if the number of cells obtained was less than the number seeded, or a simple inhibitory action, if the cells multiplied but at a slower rate than those in the control cultures.

Acute toxicity in mice. Swiss mice, 10 days old and weighing 3–4 g, were injected ip with a solution of the extract in DMSO diluted with an equal volume of physiological saline. Each extract was tested at two dose levels, one corresponding to approximately 100 mg dry culture (or 25 g/kg body weight), the other corresponding to a dry weight of 30 mg (7·5 g/kg). The animals were observed for 1 wk after treatment.

Results

The results obtained from a study of 50 strains are summarized in Table 1.

For the chick-embryo test, the toxicities are expressed as the number of LD_{50}s/g dry culture. Under the experimental conditions used, a large percentage of strains showed no toxicity for the chick embryo. Repeated tests on five strains (551–7, 632–1, 632–2, 632–3 and 634) confirmed a marked toxicity.

The results for the cell cultures are expressed using a five-point scale to indicate whether a dose of extract corresponding to 2·5 mg mycelial culture (dry weight)/ml cell culture was toxic (++++), inhibitory (+++, 75% inhibition compared with control growth; ++, 50%; +, 25%) or inactive (0). As was found with the chick embryo, the majority of the strains showed no toxicity and produced only moderate inhibition. However five of the strains produced toxic substances and nine showed a considerable capacity for inhibition.

The toxicity to mice is expressed according to a similar scale, ++++ indicating that the majority of the animals died after administration of the weaker

dose of the extract (equivalent to 7·5 g dry culture/kg), and 0 indicating that all survived after administration of the stronger dose (equivalent to 25 g dry culture/kg). Only two strains caused rapid death in all the animals, while two others showed appreciable toxicity.

The results as a whole show that about half of the strains studied were devoid of any potent toxic effects. The extracts of 15 strains were virtually free of toxicity for the mouse (grade 0 or +), the chick embryo (grade 10 or 20) and the cell cultures (grade 0 or +). The extracts of six strains gave very positive results (+ + + or + + + +) with only one test, but nine strains were toxic in at least two of the systems used. Only one strain gave clearly positive results with all three tests.

Where toxic activity was demonstrated, this did not correspond to the presence in the extracts of either PR toxin or other metabolites exhibiting a blue fluorescence and showing characteristics or chromatographic migration similar to those of PR toxin.

Discussion

In these experiments, three different biological systems were used to screen numerous Penicillium strains isolated from various cheeses in order to study the capacity for toxin production in moulds commonly used in the manufacture of cheese. The three techniques demonstrated a material's acute toxicity for a mammal, toxicity for rapidly growing cells in culture and capacity for inhibiting the development of the chick embryo. The results showed that some of the strains gave rise to toxic products, but the variability of response to the three tests clearly demonstrated that the effects exerted were not due to the presence of a single toxin but to several substances probably present in variable quantities in the extracts of the different strains.

Under our experimental conditions, the PR toxin of Wei *et al.* (1975) did not seem to be responsible for the toxic activities observed; it was rarely present and was not most readily detectable in the mould extracts showing the greatest toxicity.

It would be difficult at present to use the results reported here to judge the role that the toxic metabolites of Penicillium in cheeses may play in human toxicology and public health. The results of the three biological tests used in our experiments can only be considered presumptive. Those obtained in the mouse, however, can be extrapolated to a rather close approximation. It must also be noted that the doses injected, when compared with the weight of the animals, correspond to very large masses of mycelium; the extract of 100 mg dry culture/animal corresponds to 2 litres of culture/kg. A continuation of these experiments appears necessary to determine whether the strains of Penicillium that produced toxins in synthetic media may also synthesize toxins under the manufacturing conditions used in the production of cheeses.

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Review Section

SHIKIMIC ACID

B. STAVRIC and D. R. STOLTZ

Toxicology Research Division, Bureau of Chemical Safety, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, K1A OL2, Canada

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Summary—Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid; SA) occurs naturally in a variety of plant material. It is an intermediate in the formation of several aromatic compounds from carbohydrates. Because of its simple chemical structure, SA has been considered to be a relatively innocuous chemical. In a recently published report, however, Evans & Osman (*Nature, Lond.* 1974, **250**, 348) claimed that one of the two toxic and carcinogenic constituents isolated from bracken fern (*Pteridium aquilinum*) was SA. They also reported that commercial SA had a very potent carcinogenic effect in mice. Because of the renewed interest in this compound, some of its chemical and biological properties and its toxicology and distribution have been reviewed, together with analytical methods for its identification and quantitation. Some preliminary observations on mutagenicity tests with SA are also included.

Introduction

Shikimic acid (SA), a naturally occurring acid in a variety of plant materials, has been considered to be a relatively innocuous chemical. It is widespread in plants, some of which are consumed daily by man without any noticeable ill-effects. The chemical structure of SA does not suggest that it is a potential toxin, even less a carcinogenic compound. Nevertheless, in a recently published report, Evans & Osman (1974) claimed that one of the two toxic and carcinogenic constituents of bracken fern (*Pteridium aquilinum*) was SA.

For more than 10 years, reports have been accumulating of studies in which fresh or dried bracken fern has been found to induce intestinal, bladder and pulmonary tumours in a variety of laboratory and domestic mammals (Evans, 1968; Evans & Mason, 1965; Evans, Widdop, Jones, Barber, Leach, Jones & Mainwaring-Burton, 1971; Hirono, Fushimi, Mori, Miwa & Haga, 1973; Pamukcu, Price & Bryan, 1970; Schackam, Philp & Gowdey, 1970). Isolation of the active principle from the bracken fern was a subject of scientific interest for some time (Leach, Barber, Evans & Evans, 1971; Takatori, Nakano, Nagata, Okumura, Hirono & Shimizu, 1972; Wang, Pamukcu & Bryan, 1973), and the communication by Evans & Osman (1974) was the first report that one, of at least two, carcinogenic constituents of bracken had been identified. Although information on the solubility of SA suggests that the extraction procedures followed by Evans, Evans, Thomas, Watkins & Chamberlain (1958) and Pamukcu *et al.* (1970) to produce fractions from crude bracken for cancer testing would have retained SA, one investigator indicated that he could not identify SA in his carcinogenic fractions of bracken (G. T. Bryan, personal communications 1975). On the other hand, Saito, Umeda, Enomoto, Hatanaka, Natori, Yoshihira, Fukuoka & Kuroyanagi (1975) separated SA, quinic acid and a mixture of pterosins and pterosides (sesquiterpenes with an 1-indanone nucleus) from a bracken extract following the procedure of Leach *et al.* (1971).

After identifying SA in the carcinogenic fraction of bracken, Evans & Osman (1974) tested the carcinogenicity of commercially available SA and found it relatively active. The carcinogenic potency of SA, as reported by Evans & Osman (1974), is so startling that it justifies further investigation into the toxicological properties of this compound. The object of this review is to outline some of the pertinent information regarding SA, such as methods for its detection and its distribution, metabolism and toxicity.

General properties

SA (Fig. 1), 3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid (molecular weight 174·15, $C_7H_{10}O_5$), is a white solid with m.p. 190–191°C, soluble in water (18%), partly soluble in absolute ethanol (2·25% at 23°C) and practically insoluble in ether, chloroform, benzene and petroleum ether. SA has an absorption maximum in ethanol at 213 nm, ϵ8900 (*Merck Index*, 1968). A brief infra-red spectrum of SA has been reported by one author (Henshaw, Coult & Boulton, 1962). Specific rotations of $[\alpha]_D^{20}$ for SA were reported

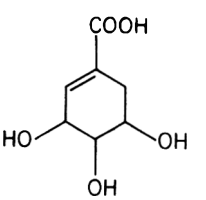

Fig. 1. Shikimic acid.

to be −177·5°C (Hulme, 1956), or $[\alpha]_D^{18}$ −183 to −188°C (*Merck Index*, 1968; Richardson & Hulme, 1955). The mass spectrum of SA obtained very recently by low-temperature mass spectroscopy did not give the expected molecular ion for SA. Instead, the molecular weight obtained was 156, which the authors atrributed to the loss of a molecule of water (Evans & Osman, 1974). With commercial SA, we obtained the same fragment of 156 which is $M^+ - 18$, using a probe temperature of 160°C.

The discovery of SA as a naturally occurring product was reported as early as 1885, when it was isolated from the plant *Illicium religiosum* Sieb. which was known to the Japanese as Shikimi-no-ki, thus leading to the trivial name for SA. Since then, SA has been shown to play a major role in the biosynthesis of essential aromatic amino acids in plants (Davis, 1955; Seifter, Rettura, Reissman, Kambosos & Levenson, 1971).

Since the chemistry and biochemistry of SA were described extensively by Bohm in a review published in 1965, these aspects of SA are not discussed in this report.

The stability of SA is not known, but there are some indications that the compound may not be very stable. Some authors suspect a spontaneous aromatization and oxidation of SA (Seifter *et al.* 1971). Although autoclaving appears not to affect the stability of SA (Davis, 1951; Davis & Mingioli, 1953), the pH of SA solutions may influence its stability.

Detection methods

Qualitative analysis

Shikimic acid is colourless and does not fluoresce under ultraviolet light. Colorimetric methods have been described for locating SA on paper chromatograms. Its chemical structure allows three types of colour indicators, i.e. tests for carboxyl function, for vicinal hydroxy groups and for a double bond (Gaitonde & Gordon, 1958; Millican, 1970; Mossor & Schramm, 1972). There are a number of chromatographic sprays for the colorimetric detection of SA, but apparently none is SA-specific. Often the same colour is produced by quinic acid, which naturally appears very frequently with SA, or by some other naturally occurring compound. Some sprays for colour reactions used in the paper-chromatographic separation of SA were reviewed by Bohm (1965). The detection limit of SA on paper chromatography, using an appropriate spray reagent is reported to be of the order of 5 μg (Gaitonde *et al.* 1958). Paper chromatography is by far the most common analytical technique used in the study of SA and the R_F values obtained with different solvent systems are listed in Table 1.

Derivatives of SA and a number of other naturally occurring organic acids, prepared with *O,N*-bis(trimethylsilyl)acetamide (Boland & Garner, 1973), hexamethyldisilazane or trimethylchlorosilane (Boudet, Alibert & Puech, 1970; Fleuriet & Macheix, 1972; Shyluk, Youngs & Gamborg, 1967), were successfully separated and identified by the use of gas–liquid chromatography (GLC). Silica gel (Morot-Gaudry, Nicol & Jolivet, 1972), ion exchange resins (Harata, Osanai & Okamoto, 1972; Jones & Barnes, 1967; Kinzel, 1962; Martinsson & Samuelson, 1970; Palmer & List, 1973), celite (Carles & Alquier-Bouffard, 1965) and other columns were also used to isolate and identify SA in various plant materials.

A limited number of investigators have used thin-layer chromatography (Hiller, 1965; Morot-Gaudry *et al.* 1972), or high-pressure liquid chromatography (Palmer & List, 1973) to separate and identify extracted SA.

Quantitative analysis

All colorimetric assays can be used or adapted for the quantitative estimation of relatively pure or well separated SA. Several quantitative methods for SA in plant material are adaptations of the GLC determination procedure based on the trimethylsilyl derivates (Boudet *et al.* 1970; Fleuriet & Macheix, 1972). We found that a slight modification of the method described by Boland & Garner (1973) for the GLC determination of trimethylsilyl derivatives of SA, gave reproducible quantitative results. Caffeine was used as an internal standard and no methoxyamine hydrochloride was added. The GLC column was 5% OV-7 on 100–200 mesh Gas-Chrom Q and the temperatures of the injection port and column were 210 and 185°C respectively. The detection limit with a flame ionization detector was about 0·2 μg SA. GLC of unconverted SA was also possible on the same column, but the detection limit was about 100 times less.

Another method for quantitative determination of SA in most tissue extracts, with the exception of liver and muscle, involves separation of SA from various interfering compounds by paper chromatography, followed by its elution and quantitative determination by a colorimetric assay (Gaitonde & Gordon, 1958).

Some quantitative methods determine the SA content by titration with dilute sodium hydroxide, after separation from other acids on a Dowex-1 × 8 column (Johnston & Hammill, 1968; Jones & Barnes, 1967). In one such method, the main sequence of the procedure was acidified-water extraction, lead precipitation, gradient elution column chromatography (Dowex-50 × 8 followed by Dowex-1 × 8) and then separation by paper chromatography and titration of the separated and eluted band of SA with dilute sodium hydroxide (Markakis, Jarczyk & Krishna, 1963).

Determination of shikimic acid in foods

Recently, a method has been described for the quantitative determination of 15 organic acids (including SA) commonly occurring in foods (Palmer & List, 1973). This method needs specially designed equipment for liquid chromatography with two columns (e.g. a pre-column injection system, a pre-column pump and a differential refractometer for detection) and the use of a fine, spherical, strongly basic anion-exchange resin. Results from the preliminary studies have been very encouraging for the determination of naturally occurring organic acids in fruit juices, wine, vinegar and other liquids. Further studies are under way to establish the precision and accuracy of the method in a variety of other food samples.

Distribution

SA has been reported to occur in variable amounts in different organs of various plants obtained from

Table 1. *Paper-chromatographic behaviour of shikimic and quinic acids**

Solvent system†	R_f value‡ × 100	
	SA	QA
Ethanol-water (75:25)	52	—
Ethanol-amyl alcohol-M-acetic acid (2:1:1)	65	—
n-Propanol-NH_4OH-water (75:1·5:23·5)	16	—
n-Propanol-acetic acid-water (75:1·5:23·5)	71	—
n-Propanol-35% NH_3-water (6:3:1)	44	48
Isopropanol-water (3:2)	68	57
Isopropanol-pyridine-water-acetic acid (8:8:4:1)	78	50
n-Butanol-formic acid-water (50:2·5:10)	43	—
n-Butanol-formic acid-water (20:1:4)	43	—
n-Butanol-acetic acid water (6:1:2)	77	—
n-Butanol-acetic acid water (50:1·5:12·5)	38	—
n-Butanol-acetic acid water (50:3:10)	37	—
n-Butanol-acetic acid water (50:3:12·5)	42	—
n-Butanol-acetic acid water (4:1:5)	46	24
tert-Butanol-M-acetic acid (3:1)	45	—
tert-Butanol-formic acid-water (70:15:15)	48	—
tert-Butanol-benzyl alcohol-isopropanol-water (1:3:1:1)	42	23
tert-Butanol-88% formic acid-water (2:1:1)	75	—
n-Amyl alcohol-5 M-formic acid (1:1)	19	—
Ethyl acetate-acetic acid-water (3:1:3)	50	36
Ether-acetone-acetic acid-water (6:3:3:1)	34	26
Acetic acid (2%)	84	94
Phenol-water-98% formic acid (3:1:0·04)	30	—
Phenol-water (3:1) plus 0.9% formic acid	56	43
Phenol-water (4:1)	55	—
Phenol-water (3:1)	44	—
Phenol saturated with water	40	—
Phenol-water-formic acid (3:1:1)	58	50

* Data from Bohm (1965).
† All proportions by vol.
‡ On Whatman no. 1 paper.

all parts of the world (Bohm, 1965; Carles & Alquier-Bouffard, 1962; Hasegawa, Nakagawa & Yoshida, 1957; Hollomon, Fuchs & Rohringer, 1967; Hulme, 1956 & 1958; Johnston & Hammill, 1968; Jones & Barnes, 1967; Kinzel & Walland, 1966; Kollas, 1964; Krishna, Markakis & Bedford, 1965; Markakis *et al.* 1963; Minamikawa & Yoshida, 1972). The concentration of SA appears to vary in some plant materials with the age of the plant or the type of storage, but reports on this subject are conflicting. For example, in *Iris pseudacorus* L. the concentration of SA fell from 739 μg/g fresh weight in February to 545 μg/g the following June (Henshaw *et al.* 1962). On the other hand, the SA concentration in apple peel increased as the apples ripened (Hulme, 1956). The concentration of SA was reported to be about 70 ppm in the peel of ripe apples (Hulme, 1956) and 100 ppm in fresh apples (Kollas, 1964).

Storage at low temperature (1–6°C) resulted in a rapid increase in the SA content of pepper fruits (Kozukue & Ogata, 1972). However, according to another report, the content of SA increased in apples when they were stored at 0°C for 4 months, but not when they were stored at 5°C (Harata *et al.* 1972).

SA has also been found in a number of micro-organisms such as *Escherichia coli* (Davis, 1955) and the mould *Penicillium griseofulvum* Dierckx (Simonart & Wiaux, 1960).

Metabolism in plants and animals

SA and quinic acid are intermediates in the formation of several naturally occurring aromatic compounds from carbohydrates. The "shikimate pathway" from glucose to aromatic compounds has been well documented and is represented in the following sequence: glucose → 5-dehydroquinate ⇌ quinate ⇌ 5-dehydroshikimate ⇌ shikimate → chorismate → prephenate → phenylpyruvate.

The first indication that SA played an important biological role in some biosynthetic pathways was reported by Davis (1951), who observed that SA, when added to some strains of *E. coli*, increased the growth rate of the bacteria by 62%. SA was subsequently shown to be a precursor for the benzene ring in the biosynthesis of naphthoquinone and vitamin K_2 (Cox & Gibson, 1966; Scharf, Zenk, Onderka, Carroll & Floss, 1971). The use of mutant strains of Neurospora showed SA also to be a precursor of anthranilic acid, indole, tryptophan, benzoic acid, phenylalanine, tyrosine and *p*-aminobenzoic acid (Martin, 1970; Metzenberg & Mitchell, 1958; Quick, 1931). It also appears to be a precursor of the aromatic rings in lignin (Noller, 1957). Furthermore it was reported that *Lactobacillus pastorianus* var. *quinicus*, occurring naturally in cider, was capable of converting quinic acid and SA into dihydroshikimic

acid (Carr, Pollard, Whiting & Williams, 1957; Phillips, Pollard & Whiting, 1956).

Oral administration of quinic acid or SA resulted in an increased urinary output of hippuric acid in man and old-world monkeys (Adamson, Bridges, Evans & Williams, 1970; Asatoor, Chamberlain, Emmerson, Johnson, Levi & Milne, 1967; Beer, Dickens & Pearson, 1951). Aromatization of either acid in these two species has been reported to be as high as 60%, while in 20 other species, including the new-world monkey, rat, mouse, cat, dog and others, this aromatization was in the range of 0–5% (Adamson *et al.* 1970). It has been confirmed that intestinal bacteria convert these acids into aromatic derivatives, since pretreatment with neomycin completely inhibited the aromatization (Adamson *et al.* 1970; Beer *et al.* 1951; Cotran, Kendrick & Kass, 1960). Evidence was also found for the interconversion of quinic acid and SA in cell cultures and plants (Bohm, 1965; Gamborg, 1967; Wienstein, Porter & Laurencot, 1959). However, when SA or quinic acid was substituted for phenylalanine in a phenylalanine-deficient diet for weanling rats, phenylalanine biosynthesis was not stimulated. Quinic acid was not converted to phenylalanine by the rat, while limited amounts of SA were converted to phenylalanine by the intestinal flora (Seifter *et al.* 1971). These results support the observations of other investigators that specific variations in the aromatization of quinic acid and SA are dependent on variations in the gut flora rather than on variations in the activity of enzymes in the tissues of the animals (Adamson *et al.* 1970).

Toxicology

Very little is known about the toxicity of SA, but the LD_5 of the acid in mice after ip injection was reported to be 1 g/kg (Evans & Osman, 1974).

When an aqueous solution of SA was given to 10-week-old mice of the TF1 strain (Tucks) as a single ip injection in a dose ranging from 1 to 20 mg/mouse, nine of 14 mice had cancerous or precancerous lesions after 8·5–16 months (Evans & Osman, 1974). No such lesions were found after 15 months in 57 control mice treated similarly with "inert materials".

The induction of dominant lethal mutations by SA in mice was investigated by the same authors. When SA was given ip or orally to a group of five male mice, each of which was mated with groups of four virgin females, a high percentage of dead implantations was observed. Orally administered SA produced a maximum of 13·6% dead implantations, while ip treatment produced a peak of 22·1%, compared with 4·4% dead implantations in the controls averaged over the 8-week period (Evans & Osman, 1974). The percentage of dead implantations was raised over most of the test period, indicating an unusually broad stage of spermatogenic susceptibility.

Even if SA were confirmed as toxic or carcinogenic in rats, there is no evidence yet that this compound is hazardous to man. Nevertheless the toxic effects of SA demonstrated by Evans & Osman (1974) were sufficiently unexpected to warrant more work with SA and to make the confirmation of these findings by another source very important. Since large segments of the population are exposed daily to SA, more comprehensive tests of its toxicity, mutagenicity and carcinogenicity in different animal species are desirable (*Lancet*, 1974).

To this end we have examined the ability of SA to revert histidine auxotrophs of *Salmonella typhimurium* as an indication of mutagenicity and carcinogenicity (Ames, Durston, Yamasaki & Lee, 1973). No genetic activity was detected using strains TA 1535–1538 with or without metabolic activation. More extensive testing for mutagenicity was similarly negative (E. Zeiger, personal communication 1975).

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BOOK REVIEWS

Fenaroli's Handbook of Flavor Ingredients. Edited by T. E. Furia and N. Bellanca. 2nd Ed. CRC Press, Cleveland, Ohio, 1975. Vol. I, pp. vii + 551, $32.95. Vol. II, pp. x + 928, $43.95.

The scope and complexity of the science and technology associated with flavour ingredients are in inverse proportion to the literature resources available in this area. Steffen Arctander's works are in a class by themselves as far as interest, nay fascination, is concerned, much in the way that old-time devotees of Beilstein found themselves browsing on and on, far beyond their immediate objectives. Bedoukian's *Perfumery and Flavoring Synthetics* is a fine, solid work, with the virtue of compactness; but the second edition is now almost 10 years old. High time, then, for a new edition of that old favorite and sturdy standby, Giovanni Fenaroli.

Expanded to well-nigh twice its original length, the new edition is impressive for its up-to-date coverage of both the old and new aspects of flavours. Experts have contributed some outstanding chapters. There is hardly need to stress the importance of human taste perception, and H. L. Meiselman's contribution on this subject is a manual in itself. To those interested in the naturally-occurring flavours in food, there is a feast in store: 85 pages on pyrazines, 96 on sulphur-containing compounds and their precursors, and 11 on thiazoles. Continuing these excellent accounts are new chapters on bread flavour and meat flavour in Volume II, as well as a discussion of the role of enzymes in food flavours. Taken with the updated tables and monographs (including FEMA lists for 1974), these features render the new edition a credit to all concerned.

Sugars in Nutrition. Edited by H. L. Sipple and K. W. McNutt. The Nutrition Foundation. Academic Press, Inc. New York, 1974. pp. xxi + 768. $45.00.

Once in a while—and it is becoming a long while—one encounters a monograph with two unique characteristics, the first being that it deals with a subject of great importance, not covered by any other recent work, and the second that it does the job superbly. This is one such *rara avis*. Its star cast of authors, tackling a judicious range of key subjects, have made an outstanding contribution to nutrition, medicine and public health.

One can only provide here some slight flavour of the remarkable character of this book. It begins with an account of the "State of the Art, past and present", which sets the stage for what is to follow. A section on "Biochemistry and psychology of sweetness" provides much food for thought on such subjective topics as pleasantness judgements and their objective basis in terms of adenyl cyclase activity in circumvallate papillae and other structures of the bovine tongue. Sugars in food receive attention, in regard to occurrence, usage and technology. In a consideration of the digestion and absorption of sugars, emphasis is laid on adaptive responses in the intestinal epithelium and gut flora. The metabolism of sugars is particularly well done, covering polyols, xylitol, a comparison of xylitol, sorbitol and fructose, lactose and galactose. As a bonus we have a masterly account of the effects of maltose and higher saccharides on metabolic functions. The involvement of the raffinose family of oligosaccharides in the production of flatulence reveals that there is still much to be learnt about human reactions and adaptations in this area of endoturbulence.

The book then proceeds to deal with a variety of clinical disorders related to sugar metabolism, beginning with inborn errors (and a useful chapter on the metabolism and toxic effects of fructose) and ending with the oral cavity. In between, a section on the therapeutic use of sugars includes excellent descriptions of safety problems in the use of xylitol orally and parenterally. All this, and much more besides. Need we say anything further to convince the reader that this book is unique?

Progress in Chemical Toxicology. Vol. 5. Edited by A. Stolman. Academic Press, Inc. New York, 1974. pp. xv + 389. $39.50.

This well-known series has established a fine reputation for excellence, interest and utility. The latest volume maintains the tradition, beginning with a comprehensive review of the absorption, distribution and excretion of drugs and poisons and their metabolites. It is appropriate that an account should follow of a system for the toxicological analysis of drugs in biological specimens.

A chapter on the phenothiazines is concerned with analytical techniques for these drugs and their metabolites. The emphasis on analysis is exemplified by chapters on "Fluorescence in toxicology", "Some microcrystal tests for drugs" and "Advances in polarography as applied to toxicological analysis". These are required reading for forensic chemists and toxicologists. The only disappointment stemmed from a chapter that should have proved a highlight of the book; "Toxicology of new synthetic organic polymers used in containers for food, pharmaceutical compounds, and drinkable water" proved to be mainly a rather mundane and uninspired compilation of standard testing procedures. Alas, no sophisticated discussion of shortcomings, pitfalls or newer approaches, or even advice to the neophyte! We recommend that this important subject be reviewed adequately in some future volume. In the meantime, this volume is well worth having close at hand, to help the chemical toxicologist in the unending quest for new worlds to conquer.

Consumer Health and Product Hazards/Chemicals, Electronic Products, Radiation. Vol. I of The Legislation of Product Safety. Edited by S. S. Epstein and R. D. Grundy. The MIT Press, Cambridge, Mass., 1974. pp. xiii + 342. £7.50.

This addition to the growing collection of volumes concerned with the health implications of modern technological advance stresses the need for effective communication between scientific and regulatory bodies in devising policies for controlling consumer products. The message throughout is that existing legal restraints are inadequate to deal with toxic substances that are pervasive or mobile in the environment.

Examples of our belated recognition of hazardous industrial chemicals in the ecosystem are numerous, ranging from PCBs, phthalates, fluorocarbons and optical brighteners to the more toxic heavy metals. The toxicological status of these materials is reviewed in a chapter dealing with toxic substances, the moral being that control of toxic materials can only be effective if it is comprehensive, and if it is exercised at least as diligently at the manufacturing stage as at the time when the substance reaches the consumer. Other chapters, each by a different author, review the legislative means for dealing with these problems in the USA and summarize the Federal controls governing the chemicals present in consumer products. The latter half of the volume is concerned with exposure to radiation from electronic products and radiological services. The implication in the latter case is that the current risk associated with consumer radiological services could be diminished still further.

A major disadvantage of this volume is its discontinuity of style and the lack of flow from one chapter to the next. Despite its pragmatic approach to a subject of great public concern, the book is unlikely to attain the status of a bestseller.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 53. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1974. pp. ix + 157. DM 44.60.

The general scheme and scope of **Residue Reviews** needs no amplification here, and it would be superfluous to reiterate the achievements of the first half-century of these volumes. Volume 53 of the series, the product of a 1973 symposium, reflects the lucid presentation and detailed treatment of previous issues, but effects a degree of continuity between contributions that was lacking in many earlier numbers.

Dealing almost exclusively with "new aspects of organophosphorus pesticides", the volume describes the search for new chemicals combining high pesticidal activity with low mammalian toxicity. The first two articles are concerned with the synthesis and insecticidal evaluation of a novel series of phosphoramidothioate derivatives and phosphorus-containing imino-oxazolidines and related compounds. The value of metabolic studies in determining structure-activity relationships is thrown into sharp relief by successive chapters on the metabolism of phosphonate insecticides, on the oxidative rearrangement of phosphorothionate insecticides and on organophosphorus insect chemosterilants. There follow a comprehensive article on the metabolism and properties of the nematocide, Nemacur, and a review on structure–activity relationships among organophosphorus fungicides. A final chapter diverges from the central theme in considering the environmental impact of dalapon (2,2-dichloropropionic acid) in relation to its chemical and physical properties.

Taking into account the fact that these papers were originally presented early in 1973, the authors are to be congratulated on the high proportion of recent references cited.

Human Intestinal Flora. Edited by B. S. Drasar and M. J. Hill. Academic Press, London, 1974. pp. xii + 263. £6.50.

For a great many years, the microbial flora of the human intestine has been largely ignored by research workers, not only from the point of view of its contribution to the metabolism of food components and xenobiotics, but also in relation to its significance and activity in healthy and diseased persons. The difficulties encountered in culturing many members of the gut flora, a major impediment to earlier research into the subject, have now been overcome and the publication of this book is indicative of the recent upsurge of interest in the gut flora and the recognition of its importance in the metabolism of drugs and food additives.

The book is divided into three sections, the first of which describes the composition of the flora, with a short introduction about the limitations of studies in this field. Factors influencing and controlling the gut flora, the distribution of the bacteria and the effects of disease are discussed, and in Section II (the longest part of the book), the metabolic activity of the flora is described. The metabolism of several groups of compounds, including glycosides, nitrogen compounds, bile acids and cholesterol, is discussed in considerable detail. Other reactions, such as the metabolism of antibiotics and the hydrolysis of esters, are treated more superficially, but there is a comprehensive list of references for the reader who finds insufficient detail in the text.

In the third section, the authors tackle (on the whole successfully) the difficult subject of the significance of gut bacteria, covering such subjects as malabsorption, diarrhoeal disease and the role of bacteria in the aetiology of cancer. The final chapter, on the significance of the gut flora in normal people, is disappointingly short, but this may reflect the paucity of information on the subject rather than a lack of interest on the part of the authors.

The self-indulgence of the authors, openly admitted in their preface, in concentrating on subjects closest to their scientific hearts, threatens at times to turn the book into a detailed résumé of their published papers. Nevertheless, some selection of topics is obviously necessary for a book of this size. To have attempted a more comprehensive review of the metabolic activities of the gut flora would have resulted in an unreadable catalogue of reactions instead of this informative and enjoyable volume.

The Fate of Drugs in the Organism. A Bibliographic Survey. Vol. 2. Compiled by Société Française des Sciences et Techniques Pharmaceutiques Working Group (Chairman J. Hirtz). Marcel Dekker, Inc., New York, 1975. pp. xiv + 578. $59.50.

This is a work of reference, comprising 3000 references, some 250 pages of tables dealing with individual drugs, and an empirical formula index. The literature survey extends to 1970, with only an occasional foray into 1971. That leaves a great deal of ground to be covered in subsequent volumes.

In the meantime, however, how useful is the present compilation? It certainly provides a bird's-eye view of the 17 aspects of each drug or other compound tabulated. These 17 properties include absorption, distribution, excretion, metabolism and isolation, so that at a glance one can see what areas have been covered in each publication and the year when the work appeared (no results are presented). Thus one finds references to ascorbic acid (1950–1970) and atropine (1948–1970) which look as if they might be fairly complete. Edetic acid, on the other hand, boasts only two references, in 1960 and 1962! Iodine has almost two pages of tabulated references, and one wonders who would seek to study its fate by this means. Moreover, since the references are not in alphabetical nor chronological order, an author index would have been a real boon.

All in all, a worthy effort; but, in this age of computerized information services, an expensive anachronism.

Intracellular Protein Turnover. Edited by R. T. Schimke and N. Katunuma. Academic Press, Inc., New York, 1975. pp. xiii + 348. $16.00.

An account of a Symposium held in 1973, published by Rapid Manuscript Reproduction, should have appeared somewhat sooner than this. The proceedings covered four general areas: protein turnover in micro-organisms, regulation of enzyme levels in animal tissues, mechanisms for the regulation of protein turnover and specific problems in the turnover of proteins. The book is a sequence of brief papers that have no summary and scant discussion. The discussions at the Symposium are not presented. Thus, even topics of direct interest to the toxicologist—such as liver δ-aminolaevulinate synthetase, and the turnover of microsomal enzymes in rat liver—fail to stimulate or greatly interest the reader.

Scanning Electron Microscopy in Biology. A Students' Atlas on Biological Organization. By R. G. Kessel and C. Y. Shih. Springer-Verlag, Berlin, 1974. pp. x + 345. DM 48.50.

> "Root and leaf and house flies' wings—
> Don't Nature make some pretty things!"
>
> Anon.

This single volume of 340 pages covers a whole range of biological matter. To achieve this, the authors have selected, apparently on the basis of photogenicity, material ranging from simple unicellular organisms to mammalian tissues.

The first chapter is devoted to the theory and operation of the scanning electron microscope and techniques for preparing specimens. Each of the remaining chapters contains a short introduction to the types of organism to be examined in its numerous photomicrographs, which, as one would expect in a book of this type, are technically magnificent.

As a picture-book of the surface of a few biological specimens, this publication is excellent, but for each specimen included, a thousand more are missing. To the student in biology, the photomicrographs are essentially snap-shots and offer less information than a set of good drawings, particularly since the latter can be modified to show hidden detail.

The volume is elegantly produced and photographically beautiful, but seems destined for the shelves of high school and university libraries.

BOOKS RECEIVED FOR REVIEW

Developmental and Genetic Aspects of Drug and Environmental Toxicity. Proceedings of The European Society of Toxicology. Vol. XVI. Edited by W. A. M. Duncan, L. Julou and M. Kramer. Proceedings of the Meeting held at Carlsbad, June 1974. Excerpta Medica, Amsterdam, 1975. pp. vii + 332. Dfl. 98.00.

Toxicologie du Styrène Monomère. Recherches Expérimentales et Cliniques chez l'Homme. By M. Oltramare, E. Desbaumes, C. Imhoff et W. Michiels. Editions Médecine et Hygiène, Genève, 1974. pp. iv + 100. Sw. fr. 12.00.

The Mammalian Kidney. Biological Structure and Function 5. By D. B. Moffat. Cambridge University Press, London, 1975. pp. viii + 263. £9.00.

Chemicals in Food and Environment. Edited by M. Webb. British Medical Bulletin, Vol. 31, no. 3. Medical Department, The British Council, London, 1975. pp. 86. £3.00.

Our Polluted Food. A Survey of the Risks. By J. Lucas. Charles Knight & Co. Ltd., London, 1975. pp. xi + 237. £4.95.

The Search for Pure Food. A Sociology of Legislation in Britain. By I. Paulus. Martin Robertson & Co. Ltd., London, 1974. pp. 144. £3.25.

Consumer Health and Product Hazards/Cosmetics and Drugs, Pesticides, Food Additives. Vol. 2 of The Legislation of Product Safety. Edited by S. S. Epstein and R. D. Grundy. The M.I.T. Press, London, 1975. pp. xi + 389. £7.50.

Fd Cosmet. Toxicol. Vol. 14, pp. 151–154. Pergamon Press 1976. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

POSSIBLE EFFECTS OF AFLATOXIN CONSUMPTION BY MAN

Aflatoxin (AF) synthesized by *Aspergillus flavus* present in feed materials was discovered in 1960 to be the cause of numerous deaths among turkeys and ducks. The leading producers of AF have since been pinpointed as *A. flavus* and *A. parasiticus*, fungi which are natural contaminants of peanuts, cotton seed, soya beans, pecans, cereals, pulses, cassava and sweet potatoes (*Cited in F.C.T.* 1969, **7,** 254; *ibid* 1972, **10,** 605). Of the various forms and metabolites of AF, those designated B_1 and G_1 show a marked capacity for inducing hepatocellular carcinomas when fed to rats, while B_2, which appears not to induce liver tumours when given orally, is associated with the appearance of liver tumours after ip injection (*ibid* 1973, **11,** 168). Bösenberg (*Z. Lebensmittelunters. u. -Forsch.* 1973, **151,** 245) has pointed out four major types of effect associated with AF, namely acute liver damage, cirrhotic liver degeneration, induction of malignant liver tumours and teratogenic and genetic damage to developing animals. The mechanism of AF toxicity has still not been fully established, although a great many studies have been concerned with this aspect of the AF problem (Wogan, *Israel J. med. Sci.* 1974, **10,** 441). Moreover, although extensive studies in animals have been carried out and marked species differences have been demonstrated in the response to AF, it is still not clear just what effects this group of toxins may have in man.

Possible association with Reye's syndrome

Some evidence has linked Reye's syndrome with AF ingestion (*British Medical Journal* 1975, **3,** 662; *Nutrition Reviews* 1971, **29,** 230). This syndrome in the human child comprises encephalopathy and fatty degeneration of the viscera, including the liver, and is an acute and often fatal condition presenting with coma, convulsions, fever and respiratory distress. Dvorackova *et al.* (*Nutr. Rep. Int.* 1974, **10,** 89) have described such a syndrome in three children aged 22, 12 and 8 months, respectively, the first showing the classical syndrome and the other two a less acute form with morphological and laboratory features pointing to the same diagnosis. Diffuse fatty changes in the liver parenchyma, kidney tubules, myocardium and striated muscles, together with cerebral oedema and hypoplasia of the thymus, lymph-nodes and adrenals, were evident in the first child, while the second and third showed cerebral oedema and fatty changes in the liver, kidneys, myocardium and striated muscles. Thin-layer chromatography of samples of liver tissue from the first and third cases demonstrated the presence of AF; samples from the second child were not available for analysis. It was considered significant that during both pregnancies the mother of the last two children worked on a poultry farm where she was in daily contact with infected fodder, and the children may have been exposed to AF during gestation or breast-feeding.

Becroft & Webster (*Br. med. J.* 1972, **4,** 117) found that chromatography of liver extracts from two children who died of Reye's syndrome in New Zealand at the age of 22 and 8 months isolated a blue-fluorescing material with the R_F characteristics of AF B_1 and, less certainly, a green-fluorescing material with the R_F characteristics of AF G_1. Amla *et al.* (*Am. J. clin. Nutr.* 1971, **24,** 609) found that the feeding of a supplementary diet of peanut flour for 2–3 weeks to three children suffering from kwashiorkor was followed by the development of anorexia, abdominal distension and an increase in liver size. The flour was found to contain 300 μg AF/kg, and the investigation was therefore broadened to include 20 children who had been fed this supplement for periods ranging from 5 to 30 days. Liver biopsies taken 1–2 months after consumption of the toxic meal showed fatty infiltration and inflammatory exudation into the liver parenchyma, while after some 4 months fibrosis and cirrhosis were apparent.

The lesions closely resembled those of Indian childhood cirrhosis, a condition of obscure aetiology. In India, liver cirrhosis is the third most common cause of death in hospital among children under the age of 5 years (Chandral Seth, *Clin. Pediat.* 1972, **11,** 128). Its characteristically insidious onset, involving low-grade fever, failure to thrive, mild abdominal distension, and either diarrhoea or constipation, is followed within a few weeks by progressive liver enlargement and the subsequent development of portal hypertension, jaundice and preterminal hepatocellular failure. The connexion between this mystery disease and the activity of fungal metabolites is drawn closer by the description of an outbreak of hepatitis among Indians who consumed spoilt maize (Krischnamachari *et al. Lancet* 1975, **I,** 1061). Jaundice was the presenting feature, and was preceded by fever, anorexia and vomiting. In the adjacent states of Gujarat and Rajasthan, 397 people were affected and 106 died, following the appearance of ascites, oedema of the lower extremities and massive gastro-intestinal bleeding. Dogs that shared the food of the affected people suffered a similar illness. The maize concerned was found to be heavily infected with *A. flavus*, and some samples contained 6·25–15·6 ppm AF. The amount of AF consumed daily by the adults was estimated at 2–6 mg. Of seven serum samples examined, two were found to contain AF B_1, and this finding, together with the bile-duct proliferation seen *post mortem*, supported a diagnosis of AF intoxication.

Further support for the contention that aflatoxicosis and Reye's syndrome may be closely connected comes from the experiment of Bourgeois *et al.* (*Lab. Invest.* 1971, **24,** 206), in which young cynomolgus monkeys were fed single doses of 0·5–40·5 mg AF B_1/kg and examined either at death or when they were killed 7 days after treatment. Doses of 1·5 mg AF B_1/kg were not fatal, and did not produce unusual signs; one death occurred among the four monkeys given 4·5 mg/kg and all those given 13·5 or 40·5 mg/kg died within 67–149 hours. Vomiting was the most consistent finding, beginning after about 12 hours and persisting for up to 72 hours. Diarrhoea and convulsions affected a small number of monkeys. All fatal doses induced lethargy and depression during the 48–96 hours before death and anorexia and coughing were common in these animals. Hypoglycaemia, an increase in non-esterified fatty acids and transaminases in the blood, cerebral oedema, neuronal degeneration, bile-duct hyperplasia, liver-cell necrosis, lymphocytolysis and marked fatty degeneration of the liver, heart and kidneys occurred, presenting a syndrome closely resembling that seen in children with Reye's syndrome.

Aflatoxins and liver cancer in Africa

Some time ago Shank, *et al.* (*Fd Cosmet. Toxicol.* 1972, **10,** 51, 61, 71, 171 & 181) published an important series of papers concerned with a study of the possible relationship between the ingestion of AF-contaminated foods and the incidence of primary liver cancer in Thailand. On the African front, Alpert *et al.* (*Cancer, N.Y.* 1971, **28,** 253) examined 480 food samples collected in Uganda, either from homes or markets, and found that 29·6% contained more than 1 μg AF/kg (1 ppb), and 3·7% contained more than 1 mg/kg (1 ppm). Foods collected from homes and from markets showed no difference in AF content, which appeared to be affected neither by the type of storage nor its duration. There was good general agreement between the frequency of AF contamination of food in various districts and the hospital records of hepatoma incidence. There were no data on hepatomas from the district showing peak aflatoxicosis incidence, but the area with the second highest frequency of AF contamination (43·8%) and concentration (10·9% of samples containing more than 1 ppm) had the highest incidence of hepatoma ($15/10^5$/year). The estimated *per capita* consumption of AF here was 0·02–2·0 mg/day. Hepatoma incidence was high in those areas where high humidity and temperature, and food scarcity, favoured mould growth.

Another study, carried out in Kenya (Peers & Linsell, *Br. J. Cancer* 1973, **27,** 473), again used samples of the main meal of the day, and if possible also of the local beer. This showed a significant correlation between the estimated intake of AF and the local incidence of liver cancer. Beers, although often made from maize rejected for food, showed a consistently low level of AF. The AF-positive diets usually featured maize, millet, sorghum, pigeon peas, cabbage and yams. The statistical analysis of the data was highly sensitive, since a few undetected cases of liver cancer in the high-altitude areas, where the incidence was some four times lower than in other areas, would have upset the conclusions. This demonstrates the difficulty of such testing.

The highest reported incidence of primary liver cancer in the world occurs in Mozambique. Van Rensburg *et al.* (*S. Afr. med. J.* 1974, **48,** 2508a) have found that hospital records in the Inhambane district of Mozambique show a liver cancer rate of 35·5 and $25·4/10^5$/year for the periods 1964–68 and 1969–71, respectively, with more than twice as many cases in males as in females. More recently, analysis of a random sample of 880 meals from the same area showed that AF occurred in 9·3%, representing a mean *per capita* consumption of 15 μg AF/adult/day or 222·4 ng/kg/day. Thus, the highest primary liver cancer rate correlates with the highest known AF intake in the world.

There are, however, many idiosyncrasies in dietary habits that may affect the incidence of liver cancer among certain tribes and certain mixed populations. In a survey of the relationship between AF intake and carcinoma in Swaziland (Keen & Martin, *Trop. geogr. Med.* 1971, **23,** 44), the low-veld area produced the highest percentage of positive AF samples in groundnuts, and the high-veld area the lowest: the relative figures being high-veld 20%, middle-veld 57% and low-veld 60%. Areas with a high percentage of AF-positive samples also had a high incidence of primary liver cancer. However, a retrospective survey of primary liver cancer among workers at the Havelock Asbestos Mines showed that Shangaans had a higher incidence than Swazis, although both tribes were provided with the same ration of AF-free groundnuts. An explanation for this anomaly was offered by the fact that Shangaans powdered the nuts in wooden, fungus-infected mortars, and supplemented their rations with locally purchased groundnuts, whereas Swazis did neither of these things. Moreover, liability to develop liver cancer appears to be greater in Shangaans than in Swazis. In addition to such occult factors, those investigating the reasons behind the varied incidence of liver cancer in different areas have to take into account the possible role of nitrosamines, native medicines, pyrrolizidine alkaloids, alcohol, malnutrition and parasitic diseases in producing liver cancer. It is hardly surprising, therefore, that the influence of any single factor remains far from clear.

[P. Cooper—BIBRA]

STUDIES ON THE TERATOGENICITY OF ALKYLBENZENE SULPHONATES

Alkylbenzene sulphonates (ABS) are widely used as household surfactants. The earlier products were derived mainly from propylene tetramer but these branched dodecylbenzene sulphonates (tpABS) have now been superseded to a considerable extent by the more readily biodegradable linear alkylbenzene sul-

phonates (LAS). Estimates of human exposure to surfactant residues on dishes and utentils have ranged from 0·3 to 1 mg/day. From this and the intake of surfactant from other sources, such as toothpastes and contaminated water supplies, Swisher (*Archs envir. Hlth* 1968, **17,** 232) estimated that the daily oral intake of surfactants of detergent origin could be between 0·3 and 3 mg. Moncrieff (*Soap Perfum. Cosm.* 1969, **42,** 447) regarded this estimate as conservative, however, and considered that amounts up to 15–20 mg/day might be ingested. The maximum daily intake of detergents in the Tokyo Metropolitan Area has been calculated to be 14·5 mg, 13·8 mg of which is derived from fruit and vegetables (unpublished report of the Tokyo Metropolitan Sanitation Bureau, 1973).

A considerable amount of work has been done to investigate the oral toxicity of various ABS. In several 90-day and 2-year studies in rats, no untoward effects were observed below dose levels of about 250 mg/kg/day (Kay *et al. Toxic. appl. Pharmac.* 1965, **7,** 812; Paynter & Weir, *ibid* 1960, **2,** 641; Snyder *et al. ibid* 1964, **6,** 133; Tusing *et al. ibid* 1960, **2,** 464). One group of workers (Oser & Morgareidge, *ibid* 1965, **7,** 819) observed a slight but statistically significant increase in liver weight in rats fed 250 mg tpABS or LAS/kg/day for 3 months, but no histological change was apparent. Effects of ABS administered in doses above 250 mg/kg/day, such as gastro-intestinal irritation, severe diarrhoea and bloating and the associated impaired nutrition and weight loss (Fitzhugh & Nelson, *J. Am. pharm. Ass.* 1948, **37,** 29), appear to be attributable mainly to the material's surfactant properties. Studies in which both tpABS and LAS were tested indicate that the two types have similar toxicity (Bornmann & Loeser, *Z. Lebensmittelunters. u. -Forsch.* 1962, **118,** 51; *idem, Fette Seifen AnstrMittel* 1963, **65,** 818; Oser & Morgareidge, *loc. cit.*). No untoward effects attributable to ABS were found in reproduction studies in this series (Bornmann & Loeser, 1963, *loc. cit.*; Buehler *et al. Toxic. appl. Pharmac.* 1971, **18,** 83; Tusing *et al. loc. cit.*).

However, it has been suggested by a group of Japanese workers that ABS have teratogenic potential. These reports have appeared mainly in abbreviated form as communications to societies or in newspaper articles and interviews published in Japanese. This review has been prepared from translations of the Japanese texts and is dependent on their accuracy. This reservation aside, the data appear to be inadequate and incomplete, and there seems to be some conflict between and within different reports of the same experiment. The reports in question cover one percutaneous and two oral studies in ICR/JCL mice.

The first oral study was reported briefly by Mikami *et al.* (*Congen. Anom., Japan* 1969, **9,** 230) and in more detail by Atsuo Nagai (internal report, Department of Anatomy, Mie University School of Medicine, 1970, **18,** 61) and Hiroo Nagai (Doctoral Thesis, Mie Municipal University, 1971). Two groups of 15 animals were treated with a detergent of undefined specification in volumes of 0·021 or 0·042 ml/g on days 6, 8 and 10 of pregnancy. Evidence on the actual dose levels is conflicting in the reports available; they could be only 2·75 and 5·5 mg/kg, or as much as 104 and 208 mg/kg according to a comment made elsewhere (H. Iseki, internal report, Department of Anatomy, Mie University School of Medicine, 1972, **20,** 119). Administration was by gavage, so animals receiving a dose of surfactant when there was little food in the stomach might have experienced heightened effects. Some maternal toxicity was observed and in comparison with the nine control animals, the test groups showed an increase in foetal resorptions, a reduction in foetal weight and an increase in the incidence of cleft palate and of exencephaly. It is not clear whether any statistical evaluation was made. The numbers of animals used and the numbers of dose levels tested were below those normally regarded as necessary for the adequate interpretation of an experiment of this kind.

The second study involving oral administration again by gavage (H. Iseki, *loc. cit.*) suffered from similar defects. Four household detergents were administered in doses of 0·63 ml (or ml/kg) to groups of 14–18 mice on days 6–11 of pregnancy. Thirteen control mice were treated with 0·6 ml normal saline. The author's concluding remarks indicate that the doses were of the order of 104 mg ABS/kg. No specifications for the detergents are included in the report. Food consumption by the dams was reduced in the test groups, maternal toxicity was reported and increases in foetal resorption, abnormalities of ribs and sternebrae and a high incidence of cleft palate were observed. From this it was concluded that these neutral detergent products were teratogenic.

Data on the study involving topical application to mice are also confused. Mikami *et al.* reported to the 13th Congress of the Japanese Congenital Malformation Society (13 July 1973) that six dose levels of an unidentified detergent solution were tested on groups of three to five mice by application on days 1–13 of pregnancy to 4 × 4-cm areas of shaved skin, but results are available for only one group of animals (Mikami *et al. Teratology* 1973, **8,** 98; *Asahi Shinbun,* 26 March 1973). The observations for this group (reported as percentage results derived from the offspring of three dams!) are claimed to show high incidences of subcutaneous haemorrhage, spina bifida, cleft palate and exencephaly. Results for control groups are not given. Evidence on dose levels is again conflicting, but they appear to range from 2·75 to 459 mg/kg for the six original dose levels and the results reported were on animals apparently receiving 332·5 mg/kg. Attempts were made to estimate the systemic dose from a percutaneous absorption rate of 0·53% demonstrated in rabbits, an exercise clearly unlikely to provide a meaningful result, if only in view of species differences.

ICR/JCL mice were used in all three of the studies carried out by the Mikami group without reference to the past performance of this strain, although it appears that such data are available (Esaki & Tanioka, *Congen. Anom., Japan* 1970. **10,** 216). Omori *et al.* (*Fd Hyg. J.* 1968, **9,** 473) published a more complete study of the effects of ABS in ICR/JCL mice. Doses of 24 or 240 mg/kg/day were administered by gavage to groups of 23 animals from day 7 to 13 of pregnancy. Slight body-weight reduction and diarrhoea were seen in test dams during the treatment, but the body weights had recovered to levels compar-

able with controls by day 18. Gavage was associated with reduced food consumption, but was not thought to have interfered with placental development. At 240 mg/kg, foetal death and resorption were increased, and there was some foetal softening. The incidence of abnormalities was similar in test animals and controls, and even in the presence of maternal toxicity the development of the young was not adversely affected. Omori *et al.* (*loc. cit.*) also exposed pregnant rats to levels of 0·025, 0·1, 0·5, 1·0 or 2·0% ABS in the diet throughout pregnancy. The groups consisted of 14–16 rats, with the exception of that fed the 2·0% dietary level, which contained only five animals. The only malformation found was exencephaly in three of the offspring of one dam fed 1% ABS, but as the rest of this litter showed poor development and survival, the authors felt that this finding was not significant.

Comparable results have been obtained in other mouse strains tested by gavage. Palmer *et al.* (*Toxicology* 1975, **3,** 91) administered doses of 0·2, 2·0, 300 or 600 mg LAS/kg/day by gavage to mice and rats from day 6 to 15 of pregnancy. Litter parameters appeared to be unaffected by doses of LAS that were non-toxic or only slightly toxic to the dam (i.e. all the dose levels in rats and the two lowest doses in mice). In mice, dose levels of 300 and 600 mg LAS/kg resulted in maternal toxicity and an increase in foetal deaths and resorptions. As noted earlier, other long-term animal tests that included reproduction studies showed no teratogenic effects in rats (Bornmann & Loeser, 1963 *loc. cit.*; Buehler *et al. loc. cit.*; Tusing *et al. loc cit.*). A further teratology study in two generations of rats exposed to a mixture of tallow alkyl ethoxylated sulphate (55%) and LAS (45%) in the diet at levels providing an intake of 80, 400 or 800 mg/kg/day has now been published (Nolen *et al. Toxicology* 1975, **4,** 231). The treatment was given either continuously or on days 6–15 of each of the six pregnancies involved. No foetal toxicity or abnormalities attributable to treatment were observed. Similarly, administration of the same mixture to rabbits by gavage in doses of 50, 100 or 300 mg/kg on days 2–16 of a single pregnancy produced no increase in foetal abnormality.

Other attempts to study the teratological effects of ABS after skin application have also been reported. In a study on mice of the ICR/JCL strain, Iimori *et al.* (*Oil Chem., Japan* 1973, **22,** 807) failed to achieve pregnancy in most of their test animals, but it is unlikely that this could be attributed to the effects of the compound. Masuda *et al.* (*J. Fd Sanit.* 1973, **14,** 580) met with more success and reported that no unaccountable abnormalities were found in mice exposed to 0·5 ml of 0·85, 1·7 or 2·55% LAS applied to an area of 4 × 4 cm on the back from day 1 to 13 of pregnancy in 16–21 dams/group. This team also investigated the effects of giving LAS in daily sc doses of 0·4–50 mg/kg to ICR/JCL mice on days 7–14 of pregnancy and observed no differences between test and control animals (F. Masuda and K. Inoue, unpublished data 1974). Palmer *et al.* (*Toxicology* 1975, **4,** 171) have examined the effects of daily application of 0·03, 0·3 or 3·0% solutions of LAS on CD-1 mice, CD rats and New Zealand white rabbits. The areas treated were 2 × 3 cm in mice, 4 × 4 cm in rats and 12 × 20 cm in rabbits, the treatment periods were days 2–13, 2–15 and 1–16 of pregnancy, respectively, and the volumes of solution applied were 0·5 ml for the rats and mice and 10 ml for the rabbits. The 0·03% concentration represents the concentration of detergent in common use, and 3·0% approximates to the levels used by Mikami *et al.* (*Teratology* 1973, **8,** 98). The doses in mice were effectively ten times those in the other species. The 3% LAS concentration caused local skin reactions and a systemic response, and only one litter was obtained from mice given this treatment. It was again concluded that embryotoxicity occurred largely where there was maternal toxicity and that the litter-orientated pattern of response indicated that it was secondary to maternal toxicity.

From the above data it can be seen that a significant amount of work suggests that adverse effects on the foetus due to ABS arise only when there are also toxic effects in the dam. Apart from the reports from Mikami's group, there appears to be no evidence to suggest that untoward effects occur below overtly toxic levels. Multigeneration studies also failed to indicate any teratogenic response. In the absence of any further information on their methodology and results, the positive Japanese findings must at this stage be regarded as tentative. They may in fact represent an artefact due either to the strain of animal used, to some unidentified component of the test materials, for which no adequate specifications were provided, or to contamination of the samples tested. Other authors have failed to repeat in other mouse strains the results of the Mikami group's skin application study in mice.

In the face of these reservations, it seems unlikely that ABS products in normal use represent a hazard to the housewife.

[F. A. Charlesworth—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

AGRICULTURAL CHEMICALS

2981. Liver tumours in rats from BHC

Ito, N., Nagasaki, H., Aoe, H., Sugihara, S., Miyata, Y., Arai, M. & Shirai, T. (1975). Development of hepatocellular carcinomas in rats treated with benzene hexachloride. *J. natn. Cancer Inst.* **54,** 801.

Liver tumours have been produced in mice by a diet containing a mixture of benzene hexachloride (BHC) isomers (*Cited in F.C.T.* 1972, **10,** 720) and by the separate administration of the β- or γ-isomer (Thorpe & Walker, *Fd Cosmet. Toxicol.* 1973, **11,** 433). Other studies have shown the α-isomer to be the most potent in this respect (Goto *et al. Chemisphere* 1972, **6,** 279; Ito *et al. J. natn. Cancer Inst.* 1973, **51,** 817). However, the present paper constitutes what is apparently the first report of liver-tumour induction by α-BHC in the rat, a species generally far more resistant than the mouse to the development of such tumours.

Rats were fed for 24, 48 or 72 wk on diets containing α-BHC at levels of 500, 1000 or 1500 ppm, β-BHC at 500 or 1000 ppm, γ-BHC at 500 ppm or δ-BHC at 500 or 1000 ppm, higher concentrations of the β-, γ- and δ-isomers having previously been shown to be toxic. All the isomers reduced weight gain and increased liver weight, α-BHC having the greatest effect and δ-BHC the least. On gross examination liver tumours were seen only in the rats maintained on 1000 or 1500 ppm α-BHC for 48 or 72 wk, and there were no tumours in other organs in any group.

Histological examination of the liver revealed slight hypertrophy of the centrilobular parenchymal cells in all groups except those treated with 500 ppm δ-BHC, but nuclear irregularities in the cells in such areas were rare. In rats given 1000 or 1500 ppm α-BHC for 48 or 72 wk there were sharply demarcated areas of hyperplastic nodules containing irregularly shaped cells, some of which showed fatty change. Hepatocellular carcinomas in three of 13 rats given 1500 ppm and one of 16 rats given 1000 ppm α-BHC for 72 wk were characterized by dilated sinusoidal spaces separated by narrow cords of pyknotic, irregularly-shaped cells containing mitotic figures. However, no bile-duct carcinomas or undifferentiated tumours were seen. Non-tumorous areas of the liver in these rats showed slight bile-duct proliferation and oval-cell infiltration, but no cirrhotic changes or connective-tissue proliferation were seen in periportal areas. No nodular hyperplasia or bile-duct proliferation was evident in groups treated with other isomers, and changes in organs other than the liver were unremarkable.

2982. The non-teratogenicity of captan

Kennedy, G. L., Jr., Fancher, O. E. & Calandra, J. C. (1975). Nonteratogenicity of captan in beagles. *Teratology* **11,** 223.

Mutagenicity tests with the phthalimide fungicide, captan, in rats and mice have given conflicting results, an increase in the number of early foetal deaths being recorded in one such study (Collins, *Fd Cosmet. Toxicol.* 1972, **10,** 353) but not in another (Kennedy *et al. ibid* 1975, **13,** 55). However, results of teratological studies in rats, rabbits, hamsters and monkeys have been uniformly negative (*Cited in F.C.T.* 1972, **10,** 263) and dogs, too, have now been added to this list.

Captan was incorporated in the diet of female beagle dogs at a dose level of 30 or 60 mg/kg throughout gestation, and the dogs were subsequently maintained for an 8-wk lactation period either on the same dose levels or on a normal diet. In no case was any effect detected on maternal weight, food consumption or behaviour, conception rate, litter size or nursing ability. Pup appearance, growth, survival and skeletal development were unaffected by treatment. At termination no increase was found in resorption sites, organ weights were normal and gross and histopathological examination revealed no treatment-related effects in either mothers or offspring.

2983. Chromosome aberrations from DDT in man

Rabello, M. N., Beçak, W., De Almeida, W. F., Pigati, P., Ungaro, M. T., Murata, T. & Pereira, C. A. B. (1975). Cytogenetic study on individuals occupationally exposed to DDT. *Mutation Res.* **28,** 449.

Chromosome mutations have been induced in mice by the ip injection of DDT (*Cited in F.C.T.* 1976, **14,** 68) but addition of p,p'-DDT to human blood *in vitro* failed to increase the number of cellular aberrations (*ibid* 1974, **12,** 272). On the other hand, an increase in chromatid lesions was found in blood cultures from men occupationally exposed to DDT and other pesticides (Yoder *et al. Mutation Res.* 1973, **21,** 335).

When 25 men who had worked in direct contact with DDT in three Brazilian insecticide plants (on average for 48 hr/wk for 2·3 yr) were compared with another 25 men from the same plants who had had no direct contact with DDT, there was no significant difference between the groups in the frequency of lymphocytes showing chromosomal aberrations. The same was true when eight workers in direct contact

with DDT at one plant were compared with an external group of ten men with no history of occupational exposure to DDT.

Nor was the frequency of aberrations correlated with plasma levels of DDT, which were, however, related to the length and to a large extent also to the degree of exposure. The mean plasma level of DDT in the 25 directly-exposed workers was 1·033 μg/ml, compared with 0·378 μg/ml in the indirectly-exposed workers and only 0·029 μg/ml in the unexposed group. However, the five indirectly-exposed workers from one plant had plasma levels that did not differ significantly from those directly exposed, and when these five men were grouped with the directly-exposed workers in the three plants there was a significant increase in the proportion of lymphocytes with chromatid breaks or gaps (12·0%, against 8·8% in the indirectly-exposed men from the other two plants and 2·2% in the unexposed group).

[The number of subjects in this study was too small for any definite conclusions to be drawn, and the findings would need to be verified in a much larger survey. The value of using any workers in a pesticide plant, albeit with no direct exposure to the pesticide, as a 'control' group must also be questioned.]

THE CHEMICAL ENVIRONMENT

2984. Lead effects in the mouse kidney

Choie, D. D. & Richter, G. W. (1974). Cell proliferation in mouse kidney induced by lead. I. Synthesis of deoxyribonucleic acid. *Lab. Invest.* **30,** 647.

Choie, D. D. & Richter, G. W. (1974). Cell proliferation in mouse kidney induced by lead. II. Synthesis of ribonucleic acid and protein. *Lab. Invest.* **30,** 652.

It has been shown that a single ip injection of lead stimulates a marked increase in cell proliferation in the rat kidney (*Cited in F.C.T.* 1973, **11,** 453). The cells induced to proliferate are apparently stimulated only once and transition of stimulated cells from the resting to the synthetic phase of the cell cycle seems highly synchronized. In the papers cited above, the authors examine the effect of a single intracardiac injection of lead acetate on the synthesis of DNA, RNA and protein and on subsequent mitotic activity in the kidney tubules of female mice aged 7–10 wk, investigated by means of autoradiography and assays of the specific activity of DNA.

Doses of lead between 0·01 and 100 μg/g body weight increased DNA synthesis (measured 30 hr later), the maximum effect being produced by a dose of 5 μg/g. At this level, there was no significant change in [^{3}H]thymidine incorporation for about 20 hr after the injection and DNA synthesis began to rise rapidly only after 27 hr, reaching a peak at 33 hr, when the mean maximal specific activity of DNA was about 15 times greater than the mean control value. This was followed 6 hr later by a wave of mitosis. The increase in DNA synthesis was preceded by an increase in the synthesis of RNA and protein, which began within 2 hr of the injection of lead and was maximal at about 24 hr. The increase in DNA synthesis was inhibited by actinomycin D (0·3 μg/g, ip), a known inhibitor of RNA synthesis, and by cycloheximide (30 μg/g), an inhibitor of protein synthesis, although in the latter case there was a delayed and much reduced rise in DNA activity.

The incorporation of [^{3}H]thymidine into DNA was not associated with DNA repair, since mitosis occurred soon after. Neither was the response due to cell necrosis, which would have been detectable histologically. The authors consider that no extraordinary pathway is involved in the stimulation of DNA replication, but suggest that the high affinity of lead for sulphydryl groups results in the inactivation of the nuclear protein responsible for gene repression, thus allowing the normal process of transcription and translation to occur. They further point out that much of the new protein produced after lead stimulation is likely to consist of enzymes responsible for DNA biosynthesis.

2985. Bacterial oxidation of mercury

Holm, H. W. & Cox, Marilyn F.. (1975). Transformation of elemental mercury by bacteria. *Appl. Microbiol.* **29,** 491.

Several bacteria and fungi have been shown to be capable of methylating mercuric chloride when incubated aerobically for 7 days (*Cited in F.C.T.* 1974, **12,** 581) and the action of selected bacteria on elemental mercury (Hg) has now been investigated.

Hg was stable when incubated aerobically for 48 hr in a sterile basal salts medium containing 0·25% glucose, but the Hg concentration in the culture was reduced by about one-third over 48 hr when the medium was supplemented with 0·1% yeast extract. Addition of *Pseudomonas aeruginosa*, *P. fluorescens*, Citrobacter sp. or *Escherichia coli* to the unsupplemented medium produced a decline in Hg concentrations, with the appearance of small amounts of mercuric ions (Hg^{2+}). This transformation occurred to a much greater degree in cultures inoculated with *Bacillus subtilis* or *B. megaterium*, which were grown in yeast-supplemented medium, and resulted in the disappearance of essentially all the Hg present over the same period. In no case was the Hg quantitatively recovered as Hg^{2+}, suggesting that much was in complexed form. A proportion of the total mercury, ranging from 18·6 to 43·2%, was found to have accumulated in the bacterial cells, and concentration factors of 196 for *E. coli*, 222 for Citrobacter and 1202 for *P. fluorescens* were calculated. No methylmercury was formed by any of the cultures within 48 hr, despite the use of three species shown to have this effect in the previous study (*ibid* 1974, **12,** 581).

Oxidation of Hg was promoted even by a sterile filtrate from a 48-hr culture of *B. megaterium*, indicating that the direct action of the bacteria was not

essential. Hg killed *P. aeruginosa* and decreased the growth of *P. fluorescens*, but the growth of the other four cultures was unaffected. It thus appeared that the effects of Hg on organisms in a complex aquatic system could not be predicted accurately from a limited study such as this.

2986. Benzene not leukaemogenic in mice

Ward, J. M., Weisburger, J. H., Yamamoto, R. S., Benjamin, T., Brown, Carolyn A. & Weisburger, Elizabeth K. (1975). Long-term effect of benzene in C57BL/6N mice. *Archs envir. Hlth.* **30,** 22.

Chromosomal changes and blood dyscrasias have been reported after human exposure to benzene (*Cited in F.C.T.* 1972, **10,** 270 & 271) and evidence has been presented that the action of benzene on the bone marrow may induce leukaemia (*ibid* 1975, **13,** 673).

In an attempt to develop an animal model to investigate this possible effect, a strain of mouse, C57BL, sensitive to radiation and radiomimetic chemicals was used. Weanling male mice were injected sc with 30% benzene in maize oil, twice weekly for 44 wk and once weekly for a further 10 wk. Injections of 0·05, 0·1, 0·1 and 0·2 ml were given in wk 1–4, respectively, the 0·2 ml volume being continued for the rest of the treatment. Negative controls were injected with maize oil in a volume equivalent to half that of the corresponding test injection, and positive controls were given 1 mg butylnitrosourea in 3 ml drinking-water daily for 120 days. Another control group remained untreated.

Benzene treatment reduced weight gain, but the difference from the controls was restored after the last injection. Deaths before wk 54 were attributed to benzene toxicity. The incidence of tumours and amyloidosis did not differ significantly between the benzene and oil-control groups, with neoplasms affecting more than 50% in each group. The incidence of any particular type of tumour was low. Amyloidosis was common, and was the primary cause of death between 58 and 108 wk of age. It affected the kidney, liver sinusoids, lymphoid follicles of the spleen, and the small intestine and caecum. No leukaemia occurred in the benzene-treated mice and there was no evidence that the treatment was associated with any carcinogenic activity. Treatment with butylnitrosourea, however, produced both poorly differentiated lymphomas originating in the thymus and metastasizing to other tissues and leukaemias in a high proportion of mice, and multiple intestinal adenomas and carcinomas occurred in a few mice in this group.

The failure of benzene to induce leukaemia in mice that are sensitive to known leukaemogenic agents suggests that the development of leukaemia in man in response to benzene exposure may be a relatively rare event dependent upon a genetically determined high level of sensitivity or the involvement of some other environmental agent.

2987. *n*-Hexane on the nerves

Korobkin, Rowena, Asbury, A. K., Sumner, A. J. & Nielsen, S. L. (1975). Glue-sniffing neuropathy. *Archs Neurol.* **32,** 158.

Inhalation of *n*-hexane has been associated with polyneuropathy in industrial workers and, more recently, in glue-sniffers (*Cited in F.C.T.* 1975, **13,** 157). Another glue-sniffing case now reported is distinctive for its pathological findings.

A young man who regularly inhaled up to 2 gal of contact cement each month developed progressive weakness of the extremities and numbness and tingling of the fingers and toes some months after changing to a cement containing 27% *n*-hexane. Transient blurring of vision and impotence were also recorded, accompanied by severe impairment of positional sense and milder impairment of vibratory and tactile senses in the lower extremities. Despite switching to another cement containing no *n*-hexane, the patient experienced a worsening of his condition over the next 3 months, until he was confined to a wheelchair and was barely able to hold the glue-can to his face. He only began to improve slowly some 9 months after entering hospital.

Nerve-conduction velocities were much reduced, and had still not fully recovered 12 months after cessation of exposure. Fascicular biopsy of the radial cutaneous nerve revealed striking segmental distension of axons by neurofilamentous masses, with secondary thinning of the overlying myelin sheath, and a marked widening of the nodes of Ranvier created by retraction of paranodal myelin, a development probably responsible for the slow impulse propagation. A second case examined by the authors showed similar axonal swelling, an effect not previously reported in *n*-hexane exposure.

2988. Methyl butyl ketones and polyneuropathy

Spencer, P. S., Schaumberg, H. H., Raleigh, R. L. & Terhaar, C. J. (1975). Nervous system degeneration produced by the industrial solvent methyl n-butyl ketone. *Archs Neurol.* **32,** 219.

The polyneuropathy identified in workers exposed to methyl *n*-butyl ketone (MBK) has since been reproduced in three animal species (*Cited in F.C.T.* 1975, **13,** 403). Further data now presented confirm this ability of MBK and show that commercial methyl isobutyl ketone (MIBK) has similar, although much less severe, effects.

The latter solvent had been used, apparently uneventfully, for several years at a 10% level in a solvent mixture (also containing 90% methyl ethyl ketone) for the application of coloured inks to polyvinyl fabrics. However, in August 1972, less than a year before the outbreak of several cases of neuropathy among the workers, MBK was substituted for MIBK in the solvent mixture and therefore became the principal suspect when the trouble developed.

Rats exposed for 6 hr daily, 5 days/wk for 4 months to the high level of 1300 ppm MBK showed slight narcosis and some loss of co-ordination during the course of each exposure period, and a slow, progressive loss of weight occurred after wk 10. After 3–4 months a pronounced symmetrical foot drop developed in the hind limbs and some animals also exhibited proximal hind-limb and fore-limb weakness. On histological examination, fibre degeneration was conspicuous in the intramuscular and distal regions of the peripheral nerves (although the sciatic nerve was affected right up to the level of the dorsal root gang-

lia) and in the lumbosacral spinal cord and cerebellum. The most prominent early change was an axonal dilatation, associated with localized fibre swelling and with a secondary myelin retraction in paranodal regions. Electron microscopy revealed swollen axonal foci containing masses of neurofilaments, numerous mitochondria and sparse neurotubules, continuous with axonal regions of more normal appearance. Myelinated fibres contained glycogen-filled mitochondrial remnants and adaxonal Schwann-cell invaginations compartmenting abnormal axoplasmic organelles. In distal and intramuscular regions, a few of the many degenerate fibres had been replaced by clusters of regenerating axonal sprouts.

When rats were similarly exposed to the even higher level of 1500 ppm commercial MIBK for 5 months, they displayed slight narcosis but gained weight normally, and there were no signs of neurological dysfunction. Examination of the central nervous system and the proximal parts of the peripheral nervous system revealed nothing remarkable, but the most distal portions of the tibial and ulnar nerves showed many axons containing large numbers of dilated, glycogen-filled mitochondrial remnants, adaxonal Schwann-cell invaginations and rare focal swellings. The MIBK used contained 3% MBK as an impurity, and further studies are now in progress with pure MIBK.

[There is a remarkable similarity between the effects of MBK ($CH_3.CO.[CH_2]_3.CH_3$) and *n*-hexane ($CH_3.[CH_2]_4.CH_3$), as described above, but whether this is due to some common metabolite has yet to be ascertained. Work is also needed to define a safe level for occupational exposure to MBK, currently set at 100 ppm. However, the present study does not suggest grounds for concern over the threshold limit value of 100 ppm for MIBK.]

2989. Trichloroethylene metabolism in man

Cole, W. J., Mitchell, Rosemary G. & Salamonsen, R. F. (1975). Isolation, characterization and quantitation of chloral hydrate as a transient metabolite of trichloroethylene in man using electron capture gas chromatography and mass fragmentography. *J. Pharm. Pharmac.* **27,** 167.

In both man and rat, trichloroethylene (TCE) is metabolized to trichloroethanol and trichloroacetic acid, and in addition chloral hydrate (CH) has been found in the blood of rats made to inhale TCE (*Cited in F.C.T.* 1973, **11,** 1154; *ibid* 1974, **12,** 163). The present study now reports the identification of CH as a transient metabolite of TCE in man.

The blood of patients anaesthetized with TCE was subjected to electron-capture gas chromatography. After about 5 min of anaesthesia, a small peak was observed with a retention time identical to that obtained with CH and chloral (to which CH decomposes on heating). Confirmatory results were obtained when three other gas-chromatographic systems were used. Gas-chromatographic analysis of blood samples taken at 5-min intervals from a patient exposed for 10 min to 1% TCE revealed that CH concentrations in the plasma rose rapidly to a peak of about 175 ng/ml in the first 40 min, after which a swift decline was observed, only trace amounts being present after 2 hr. Mass fragmentography provided further evidence for the presence of CH, peaks being obtained at precisely the same retention time after injection of a standard solution of CH, an extract of plasma to which CH had been added, and a plasma extract from patients anaesthetized with TCE.

2990. Occupational exposure to fibreglass

Enterline, P. E. & Henderson, V. (1975). The health of retired fibrous glass workers. *Archs envir. Hlth* **30,** 113.

Very fine glass fibres, of diameter up to 3 μm, have been shown to be capable of inducing mesotheliomas when implanted directly into the pleura of rats (*Cited in F.C.T.* 1974, **12,** 591). It has also been suggested recently that exposure to fibres of diameter below 3·5 μm at one pilot plant may have been associated with the development of respiratory cancer in four workers (*New Scientist*, 3 April 1975, **66,** 19). However, more extensive surveys of workers in the industry as a whole, where fibres of diameters in the 0·5–25 μm range are normally encountered, have produced evidence of no effects other than mechanical irritation of the upper respiratory tract and skin (*Cited in F.C.T.* 1973, **11,** 1145). The present study now provides further data of a generally reassuring nature, at least as far as cancer induction is concerned.

The mortality and morbidity rates among 416 men who retired in the period 1945–1972 from six plants engaged primarily in the production of fibreglass insulation, and who had survived to an age of at least 65 yr, were compared with the rates for white males in the United States as a whole. The mean follow-up period from first exposure was about 30 yr. Among the 416 there were 111 deaths, which when classified by age compared favourably with the rate in the general population. An examination of causes of death revealed no important excesses, except perhaps in the case of respiratory disease; this appeared to be slightly increased in men who had retired normally at 65, although not in those who had retired prematurely for health or other reasons. However, no mesotheliomas were recorded, and death rates from all cancers were low. In 127 men who had retired early because of ill-health, including 35 of the original 416 who had reached the age of 65 yr, there was a possible excess only in the incidence of chronic bronchitis (three cases against 0·5 expected) but the numbers were too small to allow any definite conclusions to be drawn.

[The size of fibres to which the men were exposed was not established in this work, but should obviously be taken into account in future epidemiological studies of this nature.]

2991. Haemolytic effects of PVC dust

Richards, R. J., Desai, Rashmibala, Hext, P. M. & Rose, F. A. (1975). Biological reactivity of PVC dust. *Nature, Lond.* **256,** 664.

Most readers will by now be aware of vinyl chloride (VC), and the concern that has been expressed

about its biological effects (*Cited in F.C.T.* 1975, **13**, 275). Consideration of VC as an industrial hazard has been concerned mainly with the gaseous monomer. However, workers can also be exposed to its polymers (PVC) in the form of a dust. So far, little detailed information has been available on the effects of this dust when it is inhaled or ingested.

Haemolysis tests have been used to assess the ability of other biologically active dusts to induce membrane damage. The present paper describes the use of this technique to compare the effects of PVC dusts with those of a UICC standard reference sample of chrysotile asbestos A, a very reactive material. The two finely divided dried PVC dust samples (KM 1) and (KM 2) both came from standard manufacturing processes. Sample KM 1 was found to be strongly haemolytic at fairly low concentrations (10 mg dust in an incubation mixture of 1 ml 1% erythrocyte suspension in 3 ml buffered saline causing 100% haemolysis), while KM 2 had virtually no effect. A sample of 100 mg KM 2 was required to produce a haemolytic effect equivalent to that of 1 mg KM 1. In a comparison of the activity of KM 1 with that of asbestos, total lysis was effected by a 7·5 mg sample in each case, but while this stage was reached in about 20 min with the asbestos, about 1 hr was required with the PVC. Thorough washing of KM 1 samples resulted in a 60% loss of activity after the first wash, with further decreases after subsequent washes. Testing of the washings for haemolytic activity showed that the surface-associated material, when removed from the PVC and diluted, had very little haemolytic activity.

The effect of PVC dust (KM 1) on lung-fibroblast cultures was also studied, but no significant result was obtained.

The authors conclude that some PVC dusts may constitute an industrial hazard because of the pronounced haemolytic effects associated with some readily-soluble agent aggregated on the surface of the particles. Analysis of both KM 1 and KM 2 showed immeasurable quantities of VC monomer (<1 ppm), suggesting that the soluble surface-associated material on KM 1 dust is some other chemical resulting from processing. This may open a new chapter in the VC saga.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2992. The skin and trichlorocarbanilide

Black, J. G., Howes, D. & Rutherford, T. (1975). Skin deposition and penetration of trichlorocarbanilide. *Toxicology* **3**, 253.

Last year, the FDA OTC review panel considered trichlorocarbanilide (TCC) and concluded that it should be permitted for use for 1 yr (from publication of a promised monograph), only as an antimicrobial agent in bar soap at a level not exceeding 1·5%, pending submission of further data (*Federal Register* 1974, **39**, 33124). The Panel based its conclusions partly on a series of assumptions, derived from earlier studies, about TCC's absorption through and substantivity on the skin. The present authors have already published a report of their autoradiographic method for studying the localization and distribution of germicides in the skin (*Cited in F.C.T.* 1970, **8**, 335).

This paper describes a more detailed study of TCC adsorption on guinea-pig and human skin, using 0·08% [^{14}C-carbonyl]TCC in 8% (w/v) aqueous suspensions of conventional soap, superfatted soap and a non-soap detergent containing 10 or 30% sodium alkoyl isethionate or in dimethylformamide (DMF). Samples of 0·5 ml were applied to clipped areas of 20 cm^2 on the backs of three or five male guinea-pigs and rubbed in for 2 or 10 min with a gloved finger. Animals (three/group) were also exposed twice daily for 4·5 days to examine the effects of repeated exposure. They were killed 24 or 48 hr or 2 or 4 wk after treatment, being kept in metabolism cages until death. The excreta, treated skin (punch autopsies), liver, depot fat and blood were sampled for radioactivity, and autoradiography was carried out on the skin sections.

It was found that only small amounts of TCC penetrated through to the dermis, deposition being limited mainly to the stratum corneum. There were slight variations in deposition depending on the vehicle. TCC in DMF was more evenly distributed over the skin surface than TCC applied in superfatted soap, and significantly less TCC was deposited from the 30% detergent suspension than from the other vehicles. TCC was fairly persistent up to 48 hr after application to the skin, but after 2 wk it could only be found in the stratum corneum when applied in superfatted soap or DMF and in the dermis when applied in DMF. There did not appear to be any accumulation after repeated applications.

Human volunteers were treated with 0·25 ml of a 0·08% suspension of [^{14}C]TCC in conventional soap or in 10% non-soap detergent applied to a 20-cm^2 area of the lower back. Pairs of skin biopsies were taken at 10 min, 2 days and 4 days after a single wash, and 10 min, 2 days and 2 wk after six washes applied over 3 days. One biopsy was used for autoradiography and the other was separated into dermis and epidermis and the radioactivity was determined by a combustion train technique. Very much smaller amounts of TCC were deposited in human than in guinea-pig skin and the level present decreased more rapidly with time. TCC was deposited in the stratum corneum more readily from the soap vehicle than from the non-soap detergent.

Analysis of the tissues of treated guinea-pigs for the presence of TCC revealed mean levels below 0·01 μg [^{14}C]TCC/g in the lymph nodes, liver and blood, no readings more than 10 cpm above the background of 25–30 cpm being obtained. Of the 400 μg [^{14}C]TCC administered in each wash, amounts of the order of 8 μg were found in the excreta. These small quantities may have been due to contamination or to a low level of absorption, but in either event these findings indicate that, at most, only very limited amounts of applied TCC penetrate the skin.

METHODS FOR ASSESSING TOXICITY

2993. Mutagenicity testing in the mouse

Generoso, W. M., Russell, W. L., Huff, Sandra W., Stout, Sandra K. & Gosslee, D. G. (1974). Effects of dose on the induction of dominant-lethal mutations and heritable translocations with ethyl methanesulfonate in male mice. *Genetics, N.Y.* **77**, 741.

Matter, B. E. & Generoso, W. M. (1974). Effects of dose on the induction of dominant-lethal mutations with triethylenemelamine in male mice. *Genetics, N.Y.* **77**, 753.

The first paper cited above is a study of dominant-lethal mutations induced in male mice by ip injection of ethyl methanesulphonate (EMS) in single doses ranging from 50 to 300 mg/kg. Effects on spermatozoa and spermatids were evaluated by mating the treated mice 6·5–9·5 days after the injection. Dominant lethals were detected after a dose of 150 mg EMS/kg or more, with a saturation point, at which determination was no longer accurate, of 250 mg/kg. Between these dose levels the increase in induced dominant-lethal mutations was sharp. A significant increase in the frequency of translocations was detectable in the male progeny of the treated mice even after the 50 mg/kg dose, and again rose sharply as the dose increased. This is an indication that dominant lethals, although not directly detectable, were induced at doses of 50 and 100 mg EMS/kg. It is evident that screening the male progeny of treated males for translocation heterozygosity is the more sensitive method of studying the ability of low doses of EMS to cause chromosomal damage. Since low-dose testing approximates more nearly to the usual conditions of exposure of human populations, this is an important observation, particularly since translocations represent transmissible genetic damage.

The second paper cited describes the effects of various doses of triethylenemelamine (TEM) injected into male mice. In one experiment, 0·2 mg TEM/kg was injected into males which were mated 0·5–7·5, 8·5–21·5 and 22·5–30·5 days after the dose, and in another the effect of dose on the induction of dominant-lethal mutations was studied by mating the males 4·7–7·5, 11·5–15·5 and 22·5–32·5 days after injection of a dose of TEM in the 0·035–4·0 mg/kg range. The effects on spermatocytes (matings on days 22·5–32·5) indicated true cytotoxicity, and the data for midspermatid and early spermatozoal stages (days 11·5–15·5 and 4·5–7·5, respectively) were therefore analysed. Increase of the dominant-lethal effect with dose occurred less sharply with TEM than with EMS. The ratio of genetically active dose to lethal dose was 1 : 100 for TEM compared with 1 : 3·5 for EMS, indicating that TEM has mutagenic effects at greatly subtoxic doses. The most sensitive stage in spermatogenesis for the detection of the effect was the midspermatid phase, followed by the spermatozoal and late spermatid phases in descending order of sensitivity. No induction of dominant-lethal mutations appeared to occur during the meiotic stage (matings 22·5–30·5 days after the injection).

2994. A new sensitivity test

Maurer, Th., Thomann, P., Weirich, E. G. & Hess, R. (1975). The optimization test in the guinea-pig. A method for the predictive evaluation of the contact allergenicity of chemicals. *Agents & Actions* **5**, 174.

As the incidence of allergic dermatitis has increased appreciably over the last few years, the authors cited above saw the need for an improved method of studying sensitization in animals, since other common test methods (the Draize epicutaneous test in the guinea-pig, the Magnusson and Kligman maximization test, epidermal patch tests and *in vitro* methods) either fail to account properly for the sensitivity of human skin or are insufficiently standardized. In developing their new 'optimization test', therefore, comparisons were made with results obtained with the maximization test and the Draize intradermal test conducted in a standard manner.

The three tests were carried out on guinea-pigs, using 1-chloro-2,4-dinitrobenzene (DNCB), ethyl 4-aminobenzoate (benzocaine), penicilloylpolylysin (PPL), penicillin G and 35% formaldehyde solution as test substances. Groups of 20 male and 20 female guinea-pigs were used for each experiment, 20 acting as test and 20 as control animals.

The optimization test uses Freund's adjuvant to stimulate the immune response. In wk 1, doses of 0·1 ml of the test solution without adjuvant were administered intracutaneously in the flank and back on Monday and in the back on Wednesday and Friday, and 21 hr after the treatment the animals were depilated chemically. Reactions were assessed 3 hr later. The two largest diameters of the erythematous reaction and the skin-fold thickness were measured (in mm). The 'reaction volume' (in μl) was calculated from this for each animal and each reaction. In wk 2 and 3, 0·1 ml of the test substance in adjuvant was injected into the nuchal skin three times/wk. No measurements were taken. The first challenge was given, at a fresh (flank) site, 14 days after the last induction dose in the same volume and doses as in wk 1 of the induction period (without adjuvant). After 24 hr the reactions were measured and the reaction volumes were again calculated. The reaction was assessed by calculating for each animal the average reaction volume for the first four induction doses and the standard deviation. The mean and the standard deviation were then added to give a threshold value for each animal and, if the challenge reaction exceeded this threshold value, the animal was regarded as having been sensitized. The number of positive animals in each test and control group was counted and the difference was assessed by the Fisher test. An epidermal challenge using the maximum subirritant dose was given 14 days after the intradermal challenge. A 2 × 2 cm patch of the test substance was applied under occlusion to a shorn untreated area of skin for 24 hr, and 21 hr after its removal the hair was removed chemically. The reaction was read 3 hr later by measuring any erythema and the skinfold thickness. Clearly discernible reddening of

the site was regarded as indicative of an allergic reaction.

The results of the three types of test showed the Draize test to be sensitive to potent but not to weak allergens. The optimization test gave more responsive results, the known allergens giving positive results while non-allergenic materials were negative. Weak allergens such as penicillin G, ethyl aminobenzoate and formalin gave positive results, but it is noteworthy that with penicillin G positive results were obtained only with the epidermal patch and not with the intradermal challenge. In general, the results of this test correlated well with those of the maximization test.

The authors conclude that direct stimulation of the immune system by adjuvant produces a better result in sensitization studies than the use of croton oil or sodium lauryl sulphate pretreatment or occlusive dressings to improve penetration. The optimization method described has the advantage of smoothing out variations between individual animals and providing an objective means of assessment of any reaction provoked.

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