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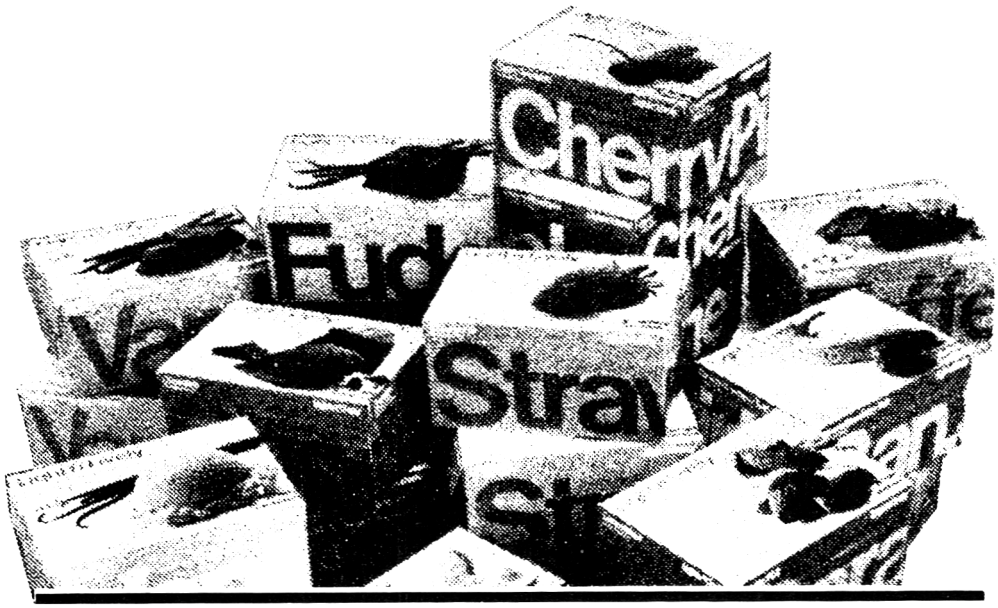

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Research Section

SCREENING OF FOOD DYES FOR GENOTOXIC ACTIVITY

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(*Received 5 June 1979*)

Abstract—Twenty-five dyes, selected from the major structural classes of synthetic food colours, currently or previously used in foods, were studied for their abilities to cause DNA damage and mutations in bacterial assay systems, with and without rat-liver microsomal activation. DNA damage, detected as significantly greater toxicity to repair-deficient *Escherichia coli* strains than to a repair-proficient strain, was induced by phloxine without microsomes and by Red 2G with microsomes. The induction of mutations was studied in liquid fluctuation tests using a tryptophan-requiring *E. coli* strain (sensitive to base substitutions) and a histidine auxotroph of *Salmonella typhimurium* (strain TA1538, specific for frameshifts). Brown FK induced base substitutions without activation, and substitutions and frameshifts after activation. Red 2G reverted the *E. coli* strain only and this specificity for base-substitution induction was verified by testing with *S. typhimurium* TA1535. The mutagenicity of Red 2G was dependent upon microsomal activation. All the other colourings screened did not induce any detectable effects. The significance of the data is discussed with respect to other information on the mutagenicity and carcinogenicity of food colours.

INTRODUCTION

An increasing number of chemicals is being shown to exhibit genotoxic effects. Levels of human exposure to such agents vary widely. Food additives may be continually ingested over long periods and therefore pose potential hazards to health.

Interest in their possible mutagenicity has been further generated by the results of short-term bacterial tests which suggest a close correlation between carcinogenicity and mutagenicity (McCann, Choi, Yamasaki & Ames, 1975; Sugimura, Sato, Nagao, Yahagi, Matsushima, Seino, Takeuchi & Kawachi, 1976). Several recent studies (Auletta, Kuzava & Parmar, 1977; Brown, Roehm & Brown, 1978; Garner & Nutman, 1977; Lecointe & Lesca, 1978; Venitt & Bushell, 1976) have been undertaken to investigate the mutagenicity of food dyes. Only a small number has proved active. In the majority of cases plate tests have been used. The interpretation of published data may be complicated by uncertainties over the degree of purity of the dye samples used.

We report here the results of using liquid screening mutation test systems with a range of synthetic colourings prepared to foodstuffs specifications. Genotoxicity was also assessed using a bacterial DNA-repair test in order to screen for compounds inducing non-mutational DNA damage. Agents causing such DNA damage can be detected as they are more toxic towards certain bacterial strains defective in the repair of their DNA than they are to repair-proficient strains of the same bacterium. Mutational changes in DNA were detected in fluctuation tests using the three tester strains *Salmonella typhimurium* TA1538 (sensitive to frameshifts), *S. typhimurium* TA1535 and *Escherichia coli* WP2 *uvrA* (specific for base-substitutions). Mutation of these auxotrophs is detected by reversion to amino-acid independence and concomitant turbid growth in minimal liquid medium. In the absence of revertants there is insufficient cell division to result in turbidity. The systems used have previously detected the activity of a sample of the monoazo food colour Red 2G in the presence of a rat-liver microsomal preparation (Haveland-Smith, Combes & Bridges, 1979).

EXPERIMENTAL

Tris-(2,3-dibromopropyl) phosphate (TRIS-BP) was obtained from Aldrich Chemical Co., Milwaukee, WI, USA, 2-acetylaminofluorene (2-AAF) and furacin (5-nitro-2-furfurylidene semicarbazone) from Koch-Light Laboratories Ltd., Colnbrook, Bucks and ethidium bromide and mitomycin C from M.W. Scientific, Eastleigh, Hants.

Rimmel Mahogany Silk (RMS) was obtained commercially; food colours were the gift of Williams (Hounslow) Ltd. (Middlesex, England); a second sample of Red 2G (Hexacol Red 2G Supra) was the gift of Pointings Ltd. (Prudhoe, Northumberland). All colourings were prepared to foodstuffs specifications. Results for Red 2G refer to the Pointings sample unless indicated otherwise. The samples of Red 2G were used without further purification. The Williams batch had a quoted dye content of 80%.

2-AAF and TRIS-BP were used in solution in dimethylsulphoxide (DMSO). Ethidium bromide, furacin, mitomycin C and all the food colours were made up in deionized water and membrane-sterilized prior to use. All the food colours were tested at their maximum sublethal concentration.

E. coli strains WP2 *trp*, WP2 *trp uvrA*, WP67 *trp uvrA polA* and WP100 *trp uvrA recA* were described by Green & Muriel (1976). The histidine-requiring *S. typhimurium* strains TA1538 and TA1535 were described by Ames, McCann & Yamasaki (1975).

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Table 1. *Structural class, legislative status and carcinogenicity of the food colours used*

Structural class	Colouring	C.I. No.	No. of countries where dye is permitted/20*	Whether permitted in EEC†	Carcinogenic status (reference)‡
Anthraquinone	Carminic acid	75470	17	+	– (Hartwell, 1951)
Azo	Amaranth	16185	20	+	?
	Sunset Yellow FCF	15985	20	+	– (Price *et al.* 1978)
	Ponceau 4R	16255	18	+	–
	Carmoisine	14720	13	+	–
	Black PN	28440	12	+	– (Drake *et al.* 1977)
	Scarlet GN	14815	9	–	–
	Ponceau 6R	16290	8	–	–
	Chrysoine S	14270	7	–	–
	Orange GGN	15980	7	–	–
	Ponceau SX	14700	5	–	+/– (Price *et al.* 1978)
	Orange G	16230	4	–	–
	Brown FK	(mixture)	3	–	– (Venitt & Bushell, 1976)
	Chocolate Brown HT	20285	3	–	–
	Allura Red AC	16035	2	–	–
	Red 2G	18050	2	–	–
	Ponceau MX	16150	NS	–	+
Azopyrollazone	Tartrazine	19140	20	+	– (Price *et al.* 1978)
	Yellow 2G	18965	3	–	–
Indigoid	Indigotine	73015	20	+	+/– (Price *et al.* 1978)
Triphenylmethane	Patent Blue V	42051	15	+	–
	Green S	44090	10	+	–
	Brilliant Blue FCF	42090	8	–	– (Price *et al.* 1978)
Xanthene	Erythrosine BS	45430	20	+	– (Price *et al.* 1978)
	Phloxine	45405	1	–	+/– (Lück *et al.* 1963; Sugimura *et al.* 1976)

NS = Not surveyed

*Number of countries in which the dye is permitted out of 20 surveyed (Bigwood *et al.* 1975).

†Colourings permitted (+) or not permitted (–) in the EEC.

‡Carcinogenic (+), non-carcinogenic (–), conflicting data (+/–) or status uncertain (?). The data is taken from Drake (1975), IARC Working Group (1975), Radomski (1974) and other sources shown in parentheses.

Details of the materials and methods used for the preparation of calcium-precipitated microsomes from the livers of phenobarbitone-induced Sprague-Dawley rats and for the screening of food dyes for genotoxic activity are described elsewhere (Haveland-Smith *et al.* 1979).

E. coli strains WP2 *trp uvrA*, WP67 *trp uvrA polA* and WP100 *trp uvrA recA* were used to test all the dyes listed in Table 1, in the DNA-repair test, both with and without microsomal activation. Ethidium bromide (167 μg), furacin (10 μg) and mitomycin C (1 μg) were used as positive controls in the DNA-repair (rec and pol) plate assays without microsomal activation. In both rec (strain WP100) and pol (strain WP67) assays, the repair-proficient (WP2) cells were treated for 3·5 hr but repair-deficient cells were treated for 5·5 and 4·5 hr respectively in the rec and pol assays before being serially diluted and plated onto nutrient agar. The plates were counted after overnight incubation at 37°C. Mutational changes in DNA were detected in fluctuation tests using *S. typhimurium* TA1538 and *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA*. The fluctuation tests were scored after 72–96 hr incubation at 37°C.

RESULTS

Agents known to induce genotoxicity were used to characterize each test system (see Tables 2, 3, 4 & 5). In the rec/pol plate assays phloxine (1 mg on each assay disc) was the only dye which induced repairable DNA damage, detected as a larger zone of inhibition of growth of the repair-defective strains in the vicinity of the disc containing the agents (the radius of the zone of inhibition of the repair-deficient strain was more than 8 mm larger than that of the repair-proficient strain after overnight incubation). All the other colourings produced no differential effects on the viability of the strains irrespective of their repair status when 1 mg of dye was used on the assay disc. The results for the positive controls are given in Haveland-Smith *et al.* (1979).

Table 2. *Results of microsomal pol assay*

Compound*	Concentration† (mg/ml)	Cs value‡		
DMSO	1 μl	1·09	1·31	0·95
TRIS–BP	0·1	*0·032*	*0·024*	*0·022*
Brown FK	10	1·34	0·91	0·98
Phloxine	10	0·27	0·43	0·38
Red 2G	10	1·26	0·74	1·20

*All the other food colours tested gave negative results in this assay.

†Concentrations are given in mg/ml except where otherwise indicated.

‡Cs = Coefficient of survival defined as percentage survival of treated repair-deficient cells/percentage survival of treated repair-proficient cells. Values in italics are considered to be positive being below 0·1.

Table 3. *Results of microsomal rec assay*

Compound	Concentration (mg/ml)*	Cs values		
DMSO	1 μl	1·09	0·96	0·82
TRIS-BP	0·1	*0·000037*	*0·000025*	*0·000062*
Brown FK	10	0·57	0·79	1·02
Phloxine†	10	0·27	0·83	0·47
Red 2G	10	*0·0011*	*0·0027*	*0·0037*
	1	0·89	0·73	0·93
Allura Red AC	10	0·61	0·93	0·89
Amaranth	5	1·10	0·76	0·93
Black PN	10	1·02	0·98	0·87
Brilliant blue	10	0·32	0·69	1·26
Carminic acid	10(%v/v)	1·50	0·86	1·12
Carmoisine	5	1·17	1·34	0·83
Chocolate Brown HT	5	1·09	0·86	0·79
Chrysoine S	5	0·87	1·21	0·86
Erythrosine BS	5	1·07	0·83	0·83
Green S	10	0·75	0·85	1·13
Indigotine	1	0·34	0·65	0·81
Orange G	10	1·81	0·79	0·93
Orange GGN	5	0·64	0·98	0·88
Patent Blue V	5	1·33	0·97	0·86
Ponceau MX	5	0·94	1·23	1·04
Ponceau 4R	10	1·33	1·08	0·86
Ponceau 6R	5	1·34	0·98	0·99
Ponceau SX	5	0·81	0·65	0·91
Scarlet GN	5	0·95	1·04	1·12
Sunset Yellow FCF	10	1·48	0·87	0·95
Tartrazine	10	0·73	0·97	0·68
Yellow 2G	5	0·70	0·86	1·07

Cs = Coefficient of survival (see Table 2)

*Concentrations are given in mg/ml except where otherwise indicated.

†In the liquid rec assay without microsomes 10 mg/ml phloxine gave a mean Cs value of *0·06*.

Values in italics are considered to be positive being below 0·1.

Table 4. *Results of fluctuation tests without activation*

Compound	Concentration (mg/ml)†	No. of turbid tubes/50, using *Escherichia coli* WP2 *uvrA* (three tests)			*Salmonella typhimurium* TA1538 (three tests)		
Furacin	0·25 μg/ml	33(14)**	36(15)**	35(15)**			
RMS	0·01%				45(19)**	40(11)**	46(16)**
Brown FK	10	33(8)**	42(16)**	39(12)**	20(18)	16(16)	17(17)
	1	27(8)**	38(16)**	31(12)**			
	0·1	9(8)	16(6)				
Phloxine	0·5				16(14)	16(18)	16(17)
	0·01	11(10)	12(12)	13(11)			
Red 2G‡	10	14(13)	14(14)	15(13)	17(17)	16(17)	14(15)
Allura Red AC	10	16(12)	16(14)	13(11)	16(13)	17(18)	18(14)
Amaranth	5	16(15)	13(13)	10(11)	14(13)	17(18)	16(17)
Black PN	10	10(12)	12(11)	11(13)	16(13)	18(18)	13(14)
Brilliant blue	10	14(11)	16(14)	12(12)	14(15)	18(18)	17(14)
Carminic acid	10(%v/v)	12(12)	11(11)	10(11)	13(13)	14(14)	11(13)
Carmoisine	5	14(12)	11(11)	9(13)	18(18)	11(14)	17(17)
Chocolate Brown HT	5	16(11)	14(13)	11(11)	11(16)	12(14)	18(17)
Chrysoine S	5	14(13)	14(11)	16(13)	16(16)	16(16)	14(14)
Erythrosine BS§	0·5	11(13)	13(14)	15(16)	14(15)	20(19)	16(18)
Green S‖	0·01	13(12)	11(11)	14(13)	15(15)	14(16)	18(16)
Indigotine	1	12(11)	16(15)	16(11)	12(14)	17(17)	16(13)
Orange G	10	13(13)	12(11)	15(15)	11(16)	16(14)	19(17)
Orange GGN	5	12(13)	16(12)	14(11)	14(14)	18(17)	11(13)
Patent Blue V	0·5	11(11)	14(12)	16(14)	19(18)	11(16)	15(17)
Ponceau MX	5	12(12)	14(15)	13(13)	15(16)	12(16)	19(14)
Ponceau 4R	10	16(13)	12(12)	15(15)	16(16)	19(16)	12(13)
Ponceau 6R	5	16(15)	9(12)	16(13)	19(16)	18(16)	18(14)
Ponceau SX	5	12(13)	10(12)	15(15)	11(14)	11(14)	14(13)
Scarlet GN	5	18(14)	16(12)	18(15)	18(18)	13(14)	14(14)
Sunset Yellow FCF	10	9(14)	16(15)	13(13)	19(18)	10(14)	13(14)
Tartrazine	10	11(14)	18(12)	11(11)	15(14)	20(14)	13(13)
Yellow 2G	5	12(14)	11(12)	11(11)	20(18)	14(14)	11(17)

†Concentrations are given in mg/ml except where otherwise indicated.

‡Red 2G (10 mg/ml) was also tested with *S. typhimurium* strain TA1535 giving negative results—the numbers of turbid tubes were 11(10) and 12(12).

§Erythrosine BS was toxic to *S. typhimurium* TA1538 at concentrations above 0·5 mg/ml; results at 1 and 5 mg/ml with *E. coli* WP2 *uvrA* were not significant.

‖Green S was toxic to *E. coli* WP2 *uvrA* at concentrations above 0·01 mg/ml; results at 0·1 mg/ml with *S. typhimurium* TA1538 were not significant and it was toxic to this strain at higher concentrations.

Values marked with asterisks differ significantly (chi-square test) from the negative control values given in parentheses (**$P < 0·01$).

Table 5. *Results of microsomal fluctuation tests*

Compound	Concentration (mg/ml)†	No. of turbid tubes/50, using *Escherichia coli* WP2 *uvrA*			*Salmonella typhimurium* TA 1538		
TRIS-BP	0·05	35(19)**	37(17)**	40(16)**			
2-AAF	0·001				35(17)**	34(14)**	37(11)**
Brown FK	10	19(7)**	25(12)**	29(15)**	47(11)**	45(11)**	49(16)**
	1	15(18)	17(16)	18(15)	33(11)**	33(16)**	30(12)**
	0·1				12(11)	16(16)	
Phloxine	0·5				18(17)	18(17)	14(16)
	0·01	16(16)	14(12)	14(14)			
Red 2G‡	10	32(13)**	34(14)**	36(13)**	19(17)	17(17)	16(15)
	1	13(13)	16(14)	13(13)			
Allura Red AC	10	19(11)	16(12)	12(14)	11(17)	18(20)	19(15)
Amaranth	5	9(12)	17(14)	13(14)	12(15)	16(20)	13(13)
Black PN	10	10(11)	12(12)	14(14)	13(17)	17(20)	13(13)
Brilliant blue	10	14(11)	14(12)	14(14)	16(17)	22(20)	12(15)
Carminic acid	10(%v/v)	15(11)	21(19)	13(12)	17(17)	17(14)	16(18)
Carmoisine	5	11(12)	11(12)	16(13)	10(10)	19(16)	14(11)
Chocolate Brown HT	5	15(11)	13(13)	13(14)	13(14)	18(20)	12(16)
Chrysoine S	5	12(11)	13(19)	15(16)	13(13)	15(10)	21(20)
Erythrosine BS§	0·5, 5	18(19)	10(12)	13(13)	9(13)	17(20)	17(16)
Green S‖	0·01, 0·1	17(17)	14(12)	12(13)	11(10)	18(20)	17(17)
Indigotine	1	9(12)	14(14)	14(13)	16(15)	17(17)	18(13)
Orange G	10	20(19)	14(17)	17(16)	10(13)	20(20)	16(17)
Orange GGN	5	13(12)	13(14)	12(13)	17(15)	20(20)	16(13)
Patent Blue V	0·5	21(19)	14(13)	16(14)	16(16)	17(15)	15(17)
Ponceau MX	5	17(16)	13(14)	10(13)	9(16)	16(15)	16(16)
Ponceau 4R	10	12(12)	16(14)	14(13)	14(16)	12(13)	18(17)
Ponceau 6R	5	11(12)	14(14)	16(14)	17(15)	17(15)	17(17)
Ponceau SX	5	13(12)	13(14)	14(14)	13(15)	18(15)	19(20)
Scarlet GN	5	12(12)	14(11)	14(13)	16(15)	11(14)	19(16)
Sunset Yellow FCF	10	12(12)	16(14)	16(13)	15(17)	15(15)	18(16)
Tartrazine	10	11(12)	16(11)	16(14)	21(17)	12(14)	16(16)
Yellow 2G	5	14(12)	15(11)	13(13)	18(17)	11(13)	15(16)

†Concentrations are given in mg/ml except where otherwise indicated.

‡Red 2G (10 mg/ml) was also tested with *S. typhimurium* strain TA1535 giving significant ($P < 0·05$) results—the numbers of turbid tubes were 21(10) and 23(12).

§Erythrosine was tested with *S. typhimurium* strain TA1538 at a concentration of 5 mg/ml; concentrations above 0·5 mg/ml were toxic to *E. coli* WP2 *uvrA*.

‖Green S was tested with *S. typhimurium* strain TA1538 at a concentration of 0·1 mg/ml; concentrations above 0·5 mg/ml were toxic to *E. coli* WP2 *uvrA*.

Values marked with asterisks differ significantly (chi-square test) from the negative control values given in parentheses.

A new liquid system (Haveland-Smith *et al.* 1979) was used to investigate the induction of non-specific DNA damage in the presence of microsomal fractions and cofactors. Agents which are negative, those which are either without effect or are toxic through a non-genetic mechanism, result in similar survivals for both repair-proficient and repair-deficient cells. This is indicated by Cs values (coefficient of survival, percentage survival of treated repair-deficient cells/percentage survival of treated repair-proficient cells) of approximately 1·0. Compounds inducing repairable DNA damage will cause a greater decrease in survival of the repair-deficient cells when compared with repair-proficient cells, resulting in Cs values of less than 1·0. Values consistently lower than 0·1 were assumed to indicate a positive effect. All the dyes were negative in the microsomal pol assay (Table 2—most data not detailed). Red 2G (at 10 mg/ml) was the only currently listed food colour that was active in the microsomal rec assay (Table 3). This confirms our earlier work (Haveland-Smith *et al.* 1979). The inability of the pol assay to detect Red 2G activity may be due to the lower sensitivity of this system, as indicated by the fact that TRIS-BP is more active in the rec assay (Tables 2 & 3).

The modified fluctuation test (see Green *et al.* 1977) was used to detect the induction of reverse mutations from amino-acid dependence to independence (prototrophy) in liquid medium. After incubation, the numbers of turbid tubes (containing prototrophic revertants) out of 50 used for each agent were scored. Spontaneous mutation was similarly measured using solvent in place of dye. A compound was considered positive only if it resulted in significantly more turbid tubes (estimated by the chi-square test) in a treated series when compared with an untreated set of tubes, in three separate experiments. The results (Tables 4 & 5) show that Red 2G specifically reverted *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* but only when activated. This suggests that Red 2G acts as an indirect base-substitution mutagen and confirms our earlier observation using a sample of dye from a different manufacturer (Haveland-Smith *et al.* 1979). Brown FK in the absence of microsomes induced base substitutions but not frameshift mutations. On addition of the microsomal activation system this ac-

tivity was lowered but the dye also induced frameshift mutations with *S. typhimurium* strain TA1538. This implies that the parent colouring, a base-substitution mutagen, may be metabolized to one or more substances acting as frameshift agents. None of the other food colours used induced detectable mutagenic damage either with or without microsomal activation (Tables 4 & 5).

DISCUSSION

The data show that under the conditions used, and with the screening methods adopted, the majority of the food colours were not genotoxic. Several reviews have been published concerning the general toxicology of food colours (Drake, 1975; Golberg, 1967; Radomski, 1974). However no general account of their mutagenicity is available.

The following azo dyes, which were inactive in the test systems described, have also been screened in Ames plate assays, with negative results: allura red (Brown *et al.* 1978), carmoisine (Brown *et al.* 1978; Garner & Nutman, 1977; Gubbini, Cardamone, Volterra-Veca, Bruzzone & Conti, 1975), Chrysoine S (Gubbini *et al.* 1975), Orange G (Garner & Nutman, 1977), Orange GGN (Brown *et al.* 1978; Gubbini *et al.* 1975), Ponceau MX (as ponceau de xylidene; Garner & Nutman, 1977), Scarlet GN, Ponceau 4R and Ponceau 6R (Gubbini *et al.* 1975), Ponceau SX (Auletta *et al.* 1977; Baker, Johnston & Barber, 1974; Brown *et al.* 1978; Price, Suk, Freeman, Lane, Peters, Vernon & Huebner, 1978) sunset yellow (Brown *et al.* 1978; Gubbini *et al.* 1975; Price *et al.* 1978). Allura red, carmoisine and Ponceau SX were non-mutagenic to several different test bacteria before and after anaerobic liquid incubation with the strains and after treatment with dithionite to effect azo-reduction (Brown *et al.* 1978). The abstract by Gubbini *et al.* (1975) does not give details of whether an activation system was used nor of what type the system may have been. Orange G and Ponceau MX were tested with *S. typhimurium* strain TA1538 only (Garner & Nutman, 1977). Several other methods have been used to test these and the other negative azo dyes. Black PN (as Brilliant Black BN), carmoisine (as Azorubine S), Orange GGN, Ponceau 4R, Ponceau 6R, Scarlet GN and sunset yellow all proved non-mutagenic in reverse and forward mutation systems in *E. coli* without activation (Lück & Rickerl, 1960). Carmoisine, Chrysoine S, Orange GGN, Ponceau 4R, Ponceau 6R, Scarlet GN and sunset yellow were also non-genotoxic to *Streptomyces coelicolor* and the fungus *Aspergillus nidulans* (Gubbini *et al.* 1975). Orange GGN, Ponceau R (probably the histological stain of Ponceau 4R), Ponceau SX and sunset yellow did not induce repairable DNA damage in a rec assay with *Bacillus subtilis* in the absence of microsomes (Kada, Tutikawa & Sadaie, 1972). Sunset yellow was not clastogenic after exposure to human peripheral lymphocytes (Zhurkov, 1975). Chocolate Brown HT has not previously been investigated for mutagenicity.

Several of the azo food colours we used were decolourized extensively by *S. typhimurium* TA1538 (data not presented). It is therefore likely that the reduction products of these dyes were present during the liquid fluctuation assays. Anilines and variously ring-substituted amino-naphthalenes are produced. However, those arising from the food colours tested would have either methyl, hydroxyl, sulphonic acid or *N*-acetyl substituents, which render them Ames-negative with TA1538 (Garner & Nutman, 1977).

The mutagenicity of amaranth (FD & C Red No. 2) has been the subject of several investigations. Interpretation of the data is confused by uncertainty as to the presence of contaminating aromatic amines and subsidiary dyes, some of which are known to be Ames-positive (Stoltz, Bendall, Stavric & Munro, 1978). Purified samples have been demonstrated to be non-mutagenic using the Ames protocol (Lecointe & Lesca, 1978). Several reports indicate negative effects of batches with either unspecified source or purity (Auletta *et al.* 1977; Baker, Johnston & Barber, 1974; Gubbini *et al.* 1975; Maxwell & Newell, 1974; Sugimura *et al.* 1976). One preparation of the dye was Ames-negative following chemical or bacterial reduction to the component amines (Brown *et al.* 1978). Likewise, a non-food-grade sample used without further purification was non-mutagenic to TA1538 (Garner & Nutman, 1977). However, another sample, marketed by the Aldrich Chemical Co. as azorubin (a synonym for amaranth; not intended for consumption), induced frameshifts in a liquid fluctuation test without microsomes (Gatehouse, 1978). Our data agree with those of Parry (1977) who found that a Williams food-grade batch of amaranth did not induce gene conversion in yeast when tested in a fluctuation assay. Amaranth has also been studied for its ability to induce mitotic recombination in yeast, dominant lethal mutations in rats and in host-mediated Ames and yeast recombination tests. No evidence for genotoxicity was forthcoming, although no data were presented (Maxwell & Newell, 1974). The situation is confused further by an FAO/WHO report (1975) citing the dye as positive in similar host-mediated assays. The fact that *in vitro* tests were negative suggested that the activity was due to metabolism *in vivo*. Cytogenetic effects of the dye have also been investigated (Maxwell & Newell, 1974). No activity was observed after *in vitro* treatment of human lung cells, but a small effect, considered to be of questionable statistical significance, was recorded using an *in vivo* assay with rat bone-marrow cells. In the absence of any data, and since unspecified dye samples were used, no further comment is possible. Amaranth was also inactive in a cytogenetic assay with human peripheral lymphocytes (Zhurkov, 1975) and in the Kada rec assay (Kada *et al.* 1972). In treat and plate tests with *E. coli* the proportion of mutants was more than doubled, but this increase was considered insignificant (Lück & Rickerl, 1960). A report suggesting a clastogenic effect of the dye (sample privately synthesized) on Chinese hamster fibroblasts was also of dubious statistical significance (Ishidate & Odashima, 1977).

The two azopyrollazone dyes, tartrazine and Yellow 2G were non-genotoxic. Yellow 2G has not previously been screened for mutagenicity. Tartrazine has been tested in various systems and found to be without effect (Brown *et al.* 1978; Gubbini *et al.* 1975; Kada *et al.* 1972; Lück & Rickerl, 1960; Price *et al.* 1978). There is, however, one record of a significant increase in chromosomal aberrations, without any exchanges, arising in human lymphocytes following *in vitro* treatment (Price *et al.* 1978).

The triphenylmethane colours, Brilliant Blue FCF, Green S and Patent Blue V proved inactive in the genetic systems used. Brilliant blue is non-mutagenic in Ames assays (Auletta *et al.* 1977; Brown *et al.* 1978; Price *et al.* 1978). Patent Blue V has been tested once before with negative results (Gubbini *et al.* 1975). Green S has not been subjected to mutagenicity testing elsewhere.

Indigotine, which was also non-genotoxic, did not induce DNA damage in the *B. subtilis* rec assay (Kada *et al.* 1972), and was non-mutagenic to *E. coli* (Lück & Rickerl, 1960), to various *S. typhimurium* Ames tester strains (Auletta *et al.* 1977; Brown *et al.* 1978; Gubbini *et al.* 1975) and to *A. nidulans* and *S. coelicolor* (Gubbini *et al.* 1975). Furthermore, the dye did not induce cytogenetic effects in human lymphocytes (Zhurkov, 1975).

The naturally-occurring anthraquinone dye, carminic acid, also inactive, has been demonstrated to be negative in several of the systems mentioned above (Barale, Fumero, Loprieno, Meriggi, Mondino & Silvestri, 1978; Brown & Brown, 1976; Kada *et al.* 1972; Lück & Rickerl, 1960). In addition, carminic acid did not induce mutation in five different markers in *Schizosaccharomyces pombe in vitro* or in a host-mediated assay (Barale *et al.* 1978).

Several different xanthene dyes have been tested for genetic effects. Of the two studied in the present investigation, erythrosine was inactive whilst phloxine induced DNA damage only in the absence of microsomes, but was neither directly nor indirectly mutagenic. The observation that erythrosine does not induce DNA damage confirms an earlier report (Kada *et al.* 1972), with the *B. subtilis* rec assay. Samples of the dye were Ames-negative (Auletta *et al.* 1978; Brown *et al.* 1978; P. J. Price, personal communication, 1978; Sugimura *et al.* 1976). However, other samples of German origin and unspecified purity, induced small but statistically significant effects in a variety of forward and reverse mutation systems in *E. coli* (Lück & Rickerl, 1960; Lück, Wallnofer & Bach, 1963). The equivocal nature of these data may be due to a requirement for photodynamic activation of the dye. In conjunction with white light erythrosine is mutagenic to *Serratia marcescens* (Kaplan, 1949), capable of inactivating transforming DNA and positive in the rec assay (Yoshikawa, Kurata, Iwahara & Kada, 1978). Erythrosine did not induce respiratory mutations in the yeast, *Saccharomyces chevalieri* (Nagai, 1976).

The colourings that were genotoxic in this investigation were phloxine, and the azo dyes Brown FK and Red 2G. Using the fluctuation test, we were unable to confirm the treat and plate data indicating that phloxine was mutagenic to *E. coli* (Kada *et al.* 1972) although our positive DNA-damage results agreed with those obtained by these workers. This discrepancy is being investigated further. Our results also suggest that phloxine may be de-activated by microsomes and this may explain why the dye was inactive in a host-mediated rec-assay (Kada *et al.* 1972). Phloxine was non-mutagenic to *E. coli* in a plate assay (Lück *et al.* 1963) and in the Ames test (Brown, Dietrich & Bakner, 1979; Sugimura *et al.* 1976). However in contrast to erythrosine, it induced yeast petite mutants (Nagai, 1976) and was a weak inducer of 8-azaguanine resistance mutations in human cell cultures (Kuroda, 1975). As with erythrosine the variation between the genotoxic data for phloxine may be due to a requirement for photodynamic activation, which may potentiate the mutagenicity (Nishioka, 1975), and the DNA-damaging capacity of the dye (Nishioka & Katsura, 1975; Yosikawa *et al.* 1978). Discrepancies may also be due to variation in the degree of halogenation of the fluorescein structure according to the sample used.

Our data show that Brown FK was mutagenic. Venitt & Bushell (1976) reported that three different samples of Brown FK were frameshift mutagens in the Ames test after microsomal activation. One sample was also directly active. Our sample induced base-substitutions prior to activation and frameshifts and transitions after incubation with microsomal extracts. It is likely that this can be explained in terms of the activities of the compounds comprising the parent dye, two of which are Ames-positive (Venitt & Bushell, 1976). It is noteworthy that, in our hands, Brown FK parent dye does not induce non-specific DNA damage. Further data concerning the genotoxicity of Brown FK and its constituents will be presented elsewhere.

The mutagenicity of Red 2G has been reported by us previously (Haveland-Smith *et al.* 1979). This observation and the induction of transition mutations are now confirmed by the demonstration that the same sample of dye is indirectly mutagenic to another tester strain (TA1535) also sensitive to base substitutions. Moreover, a batch from a different manufacturer is genotoxic with similar potency and specificity.

It is possible to assess the degree of correlation between the results of short-term mutagenicity and animal carcinogenicity testing (see Table 1) for the food colours we have screened. Although erythrosine is considered non-carcinogenic, not all the results of *in vitro* mutagenicity assays have been negative. Brown FK and Red 2G, both active in bacterial assays, are currently permitted in the UK (The Colouring Matter in Food Regulations, 1973) on the basis of the negative results of single unpublished carcinogenicity trials for each. Ponceau MX is the only colouring used for which there is definite evidence of carcinogenicity (I.A.R.C. Working Group, 1975). Nevertheless, this dye is inactive for all short-term tests in which it has been assayed. If erythrosine, amaranth and Ponceau SX are not considered because of conflicting results, 17/22 dyes tested are negative for carcinogenicity and mutagenicity. There is no case of a dye exhibiting both animal carcinogenicity and genotoxicity to bacteria. Two colourings are carcinogenic and were negative in the short-term assays and three were positive in the bacterial assays but are non-carcinogens.

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GENOTOXICITY OF THE FOOD COLOURS RED 2G AND BROWN FK IN BACTERIAL SYSTEMS; USE OF STRUCTURALLY-RELATED DYES AND AZO-REDUCTION

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Abstract—The azo food colour Red 2G has previously been shown to induce DNA damage and mutations in bacterial test systems if microsomal activation is used. This genotoxicity has been studied further by investigating the activities of two structurally-related dyes, Red 10B and Red 6B, in these assays. The effects of chemical reduction on the genotoxicities of the three dyes have also been observed. The results show that whilst Red 6B was inactive under all conditions, Red 10B was genotoxic without activation, and this activity was abolished by microsomal activation and/or chemical reduction. Reduction eliminated the indirect activity of Red 2G and did not affect the lack of activity of the dye in the absence of microsomes. Since ingested food colours may be reduced by intestinal microflora, Red 2G and Brown FK (also mutagenic) were subjected to anaerobic *in vitro* incubation with whole-cell rat caecal extracts prior to testing with or without microsomes. The indirect genotoxicity of Red 2G was reduced after caecal pretreatment. Although the direct activity of Brown FK was reduced, the indirect activity of the dye was either unaffected or slightly enhanced according to the genetic systems used. The data are discussed in relation to the structures of the dyes used, the contribution of the tester strains to reduction, and other factors.

INTRODUCTION

We have previously reported on the genotoxicities of the food colours Brown FK and Red 2G in liquid bacterial assays (Haveland-Smith & Combes, 1980; Haveland-Smith, Combes & Bridges, 1979). In this communication we present data on the genetic effects of two de-listed colours, Red 10B and Red 6B, which are structural analogues of Red 2G. The activities of the three dyes are discussed in relation to their structures.

After ingestion, food colours are known to be susceptible to metabolism by the intestinal microflora (Brown, 1977; Hess & Fitzhugh, 1955; Williams, 1959). The main effect on azo dyes is reduction to their component amines, a process which is accompanied by decolourization. We have therefore studied the effects of chemical reduction with dithionite on the subsequent direct and indirect activities of these dyes. In an effort to simulate *in vivo* conditions, Red 2G and Brown FK were anaerobically incubated with rat caecal whole cell extracts prior to testing with or without microsomes. Reduction has been observed to alter the mutagenicity of various colourings previously (Brown, Roehm & Brown, 1978). The contribution of the bacterial strains to reduction has also been considered (Garner & Nutman, 1977).

EXPERIMENTAL

2-Aminofluorene (2-AF) was obtained from the Aldrich Chemical Co., Milwaukee, WI, USA; sodium dithionite from M.W. Scientific, Eastleigh, Hants., and rutin from the Sigma Chemical Co., St. Louis, MO, USA. The colourings were the gift of Williams (Hounslow) Ltd., London and were used without further purification. Chromatography of Red 2G in a phenol-water solvent (4:1 w/v) revealed a main coloured band corresponding to the dye. Traces of two subsidiary components were detected, one as a discrete faint band the other as an ultraviolet fluorescing line. (C. N. Edwards, personal communication, 1978). 2-AF and rutin were used in solution in DMSO. The colourings were made up at concentrations of 0·1, 1 or 10 mg/ml in deionized water and membrane-sterilized prior to use.

Escherichia coli strains WP2 *trp*, WP2 *trp uvrA*, WP67 *trp uvrA pol A* and WP100 *trp uvr A rec A* were described by Green & Muriel (1976). The histidine-requiring *Salmonella typhimurium* strains TA1538 and TA1535 were described by Ames, McCann & Yamasaki (1975). Details of the materials and methods used for the preparation of calcium-precipitated microsomes from the livers of phenobarbitone-induced Sprague-Dawley rats and for the screening of food dyes for genotoxic activity are described elsewhere (Haveland-Smith *et al.* 1979).

The effects of chemical reduction with dithionite on the subsequent direct and indirect activities of Red 2G, 6B and 10B were studied. Sodium dithionite was added to the dye solution at a concentration of 0·05 mg/ml (the maximum sublethal concentration) immediately before addition of the dye to the test system.

In an attempt to simulate *in vivo* conditions, Red 2G and Brown FK were anaerobically incubated with

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rat caecal whole-cell extracts before testing with or without microsomes. The caeca were removed from three 10-wk-old-Sprague-Dawley rats into a tared beaker. Quarter-strength Ringer solution was added at 10 ml/g of caecal material which was then sliced and shaken. The sediment was allowed to settle and the supernatant decanted off. A 50-μl sample of supernatant was added to 1 ml of sterile dye solution in Sorenson's phosphate buffer, the resultant mixture being incubated anaerobically in Gaspak jars at 37°C using a Gaspak apparatus (BBL). After 2 hr, the mixture was membrane-filtered, the filtrate being then tested in the rec and fluctuation assays as previously described. For the rec assay, the repair-proficient (WP2) cells were treated with the activated dye for 3·5 hr and the repair-deficient cells were treated for 5·5 hr before being serially diluted and plated onto nutrient agar. Plates were counted after overnight incubation at 37°C.

To determine the decolourization of the dyes produced by incubation with the tester strains or with caecal extract, the dyes were incubated with or without bacteria in thioglycollate broth for 24 hr (caecal extract) or for 28 hr (*S. typhimurium* strains TA1535 and TA1538 and *E. coli* strain WP2 *uvrA*) and then observed for decolourization using a Beckman Model 25 spectrophotometer.

RESULTS

The characterization of the assay systems with positive control agents and the intrepretation of the genetic tests are described in the preceding paper (Haveland-Smith & Combes, 1980).

The effects of reduction with sodium dithionite on the genotoxicity of Red 2G and Red 10B are presented in Table 1. The results show that, in addition to Red 2G, Red 10B was also genotoxic but that Red 10B was direct acting and inactivated by the microsomal preparation under these conditions.

Dithionite pretreatment eliminated the activity of microsomally-activated Red 2G and eliminated the direct activity of Red 10B. Red 6B exhibited no activity in any of the test systems used. In this study sodium dithionite was added at 0·05 mg/ml instead of at the molar excess levels used elsewhere (Brown *et al.* 1978). High concentrations of dithionite were too toxic and only partial decolourization was observed at the sublethal levels used.

The effects of preincubation of Brown FK and Red 2G with rat caecal microflora prior to testing in the rec and fluctuation assays are shown in Tables 2 and 3 respectively. The results of testing without caecal activation in the presence or absence of microsomes are summarized in each table (for details see Haveland-Smith & Combes, 1980). In addition to standard positive control agents, rutin (quercetin-3-rutinoside) and 2-AF have been used to detect the activating capacity of the caecal extracts. The weak indirect activity of rutin with microsomes has been shown to be potentiated by the addition of gut bacterial enzyme extracts (Brown, Dietrich & Brown, 1977). Our results show that anaerobic pretreatment with rat caecal extracts containing whole cells is capable of activating rutin in the presence of microsomes to induce non-specific and mutational DNA damage. 2-AF was used as a positive control for the induction of base substitutions. This aromatic amine is known to be activated to a mutagen by extracts of intestinal bacteria (McCoy, Speck & Rosenkranz, 1977). 2-AF was mutagenic to WP2 *uvrA* only after preincubation with caecal extract followed by microsomal activation (Table 3).

Caecal extract reduced the activity of Red 2G in the microsomal rec assay (Table 2). With anaerobic preincubation with caecal extract, microsomally-activated Red 2G resulted in Cs values roughly 30-fold greater than those obtained with microsomes alone (see Haveland-Smith & Combes, 1980). Similar pretreatment decreased the mutagenicity of the dye in the microsomal fluctuation test (Table 3). Overnight incubation with caecal extract caused a complete loss of activity of Red 2G in the rec assay. Caecal extract neither activated Brown FK in the rec assay (Table 2) nor altered the mutagenicity of the dye to *S. typhimurium* TA1538 (Table 3). Anaerobic pretreatment with caecal extract did however cause a slight enhancement of Brown FK mutagenicity with *E. coli* WP2 *uvrA*, as revealed by an increase in the number of turbid tubes over the number found when the dye was tested

Table 1. *Effects of sodium dithionite on the genotoxicity of Red 2G, Red 6B and Red 10B*

Microsomal activation	Dithionite pretreatment	Genotoxicity of Dithionite (0·05 mg/ml)	Red 2G (10 mg/ml)	Red 6B (10 mg/ml)	Red 10B (10 mg/ml)
		Rec assay (Cs† values)			
−	−	0·96, 0·82	0·93, 0·97	0·76, 0·93	*0·013, 0·012*
−	+		0·96, 1·03	0·84, 0·66	0·69, 0·78
+	−	0·79, 0·88	*0·0038, 0·0033*	1·23, 0·89	0·37, 0·48
+	+		0·87, 0·83	0·79, 0·83	1·16, 0·92
		Fluctuation test with WP2 *uvr A* (no. of turbid tubes/50)			
−	−	19(14), 16(16)	14(14), 17(16)	11(10), 13(14)	34(10)**, 34(14)**
−	+		13(14), 15(16)	16(14), 12(16)	17(14), 17(16)
+	−	14(14), 16(14)	36(13)**, 35(15)**	14(14), 15(14)	18(14), 19(14)
+	+		17(14), 17(14)	16(14), 13(14)	19(14), 17(14)

†Cs = Coefficient of survival, defined as percentage survival of treated repair-deficient cells/percentage survival of treated repair-proficient cells.

Values for two tests are given in each case; those in italics are considered to be positive being below 0·1; those marked with asterisks differ significantly (chi-square test) from the corresponding negative control values given in parentheses (**$P < 0·01$).

Table 2. *Effect of preincubation of the colourings Brown FK and Red 2G with rat caecal extract on the results of the rec assay*

Agent	Concentration (mg/ml)	Results of rec assay (Cs* values) using the following activation systems*		
		Caecal extract†	Caecal extract + microsomes†	Microsomes only‡
Rutin	0·1	0·16	*0·041*	0·64
		0·23	*0·048*	0·87
		0·13	0·037	0·59
Brown FK	10	1·12	0·96	NS
		0·89	0·84	
		0·76	0·95	
Red 2G	10	1·16	*0·086*	S(*0·0025*)§
		1·03	*0·094*	
		0·91	*0·091*	
	10‖	0·87	0·39	
		1·09	0·44	
		0·93	0·61	
	1	1·06	1·02	NS
		0·78	0·74	
		0·98	0·93	

NS = Not significant S = Significant

*Cs = Coefficient of survival defined as percentage survival of treated repair-deficient cells/percentage survival of treated repair-proficient cells.

†Cells were preincubated with caecal extract only or preincubated with caecal extract followed by microsomal activation.

‡These results with the exception of that for rutin are detailed in Haveland-Smith & Combes, 1980. There were no significant results for either dye in the rec plate assay without activation.

§This is the mean value.

‖In this case the cells were preincubated with caecal extract overnight.

Values in italics are less than 0·1 and are therefore considered positive.

with microsomes only (Table 3 and see Haveland-Smith & Combes, 1980, Table 5). However, in the absence of microsomes, caecal extract reduced the significance level for the direct activity of Brown FK for WP2 *uvrA* when tested at 1 mg/ml from a significance level of $P < 0{\cdot}01$ to $P < 0{\cdot}05$ (Table 3).

Table 4 presents data on the decolourization of Brown FK, Red 2G and Black PN after incubation in reducing conditions with the tester strains used and with caecal extract. The diazo colour, Black PN, was completely decolourized after all treatments and the data are included for comparative purposes. The tester strains caused some decolourization of Brown FK but Red 2G was resistant to reduction. However both dyes were over 50% decolourized by caecal extract.

DISCUSSION

The possible mechanism of activity of Red 2G has been studied firstly by screening two structural analogues, Red 10B and Red 6B for genotoxicity and secondly by investigating the effect of azo-reduction on the subsequent activity of these three dyes. The data show that Red 10B induced DNA damage and base-substitution mutations, but, unlike Red 2G, Red 10B appeared to be direct acting, being de-activated in the presence of microsomes. The samples of Red 6B tested were not genotoxic with or without prior activation. The structures of the three dyes are shown in Fig. 1. Red 2G is the *N*-acetylated derivative of Red 10B. Red 6B possesses two *N*-acetyl groups, one on each side of the azo bridge. It may be postulated that at least one of the steps in Red 2G activation *in vitro* is *N*-deacetylation, yielding Red 10B. This reaction may occur in acid conditions (Commission of the European Communities, 1977) such as those occurring in the stomach, thus posing a potential hazard. The non-activation of Red 6B may be due to the presence of two *N*-acetyl groups which may halve the overall deacetylation rate of the molecule. Microsomal extracts with higher deacetylation activity might be able to activate this dye. Evidence exists which suggests that general deacetylation reactions do not occur as readily *in vivo* as they do *in vitro* when mediated by the microsomal systems of rats and other organisms (King & Phillips, 1969; Williams, 1959). Since deacetylation would decrease the polarity of the molecule it would be unlikely to be an *in vivo* detoxification reaction. However, Red 10B which would be formed by *in vitro* deacetylation, is direct acting and is microsomally detoxified. There are strong indications that aromatic amines require further activation for genotoxicity possibly involving *N*-hydroxylation (McCann, Choi, Yamasaki & Ames, 1975; Miller & Miller, 1969). Some may be weakly active in the absence of microsomal extracts. This may be the reason for the observed low potency of Red 10B (and Red 2G after activation), but does not explain why Red 10B is deactivated. If the microsomal extract possesses acetylating and deacetylating activities when it is added to Red 10B the 8-amino group would be acetylated, and Red 2G would accumulate if by this time the deacetylase activity had declined resulting in a low conversion rate back to Red 10B. The opposite would occur on addition of microsomes to Red 2G when the initial reaction would involve deacetylation favouring Red 10B formation.

Several investigators have found that reduction of certain azo dyes, including Brown FK, may be an activation step for general toxicity (Drake, 1975; Radomski, 1974) and for mutagenicity (Garner & Nutman, 1977; Venitt & Bushell, 1976). It is thought that azo dyes are subjected to reduction *in vivo* as a result of metabolism by intestinal bacteria yielding amines (Brown, 1977; Walker, 1970). Our results show that whole-cell caecal extracts reduced the two dyes used. Red 2G is reduced *in vitro* by extracts of the intestinal bacterium *Streptococcus faecalis* (Walker, Gingell & Murrells, 1971) and this bacterium was identified, amongst others, in the caecal extracts used in the present study. Although cell-free extracts of bacteria are generally more efficient,

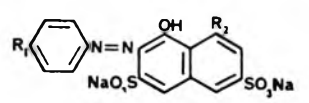

Fig. 1. The structures of Red 2G (R_1 = H, R_2 = NH·CO·CH_3), Red 6B (R_1 = NH·CO·CH_3, R_2 = NH·CO·CH_3) and Red 10B (R_1 = H, R_2 = NH_2).

Table 3. *Effect of preincubation of the colours Brown FK and Red 2G with rat caecal extract on results of fluctuation tests*

Strain	Agent	Concentration (mg/ml)	Result of fluctuation test (no. of turbid tubes/50) using the following activation systems: Caecal extract†	Caecal extract + microsomes†	Microsomes only‡	None‡
WP2 *uvr A*	2AF	0·1	18(12)	25(12)**	13(12)	9(12)
			17(14)	28(14)**	16(14)	13(14)
			16(17)	33(17)**	11(11)	12(11)
	Red 2G	10	16(15)	25(15)*	S**	NS
			17(17)	29(17)*		
			12(12)	22(12)*		
		1	15(15)	16(15)	NS	NT
			16(18)	14(18)		
			16(16)	17(16)		
	Brown FK	10	34(12)**	43(12)**	S**	S**
			33(12)**	44(12)**		
			34(14)**	47(14)**		
		1	25(14)*	31(14)**	NS	S**
			25(12)*	30(12)**		
			24(12)*	32(12)**		
TA1538	Rutin	0·01	19(16)	38(16)**	26(16)	18(16)
			19(17)	39(18)**	29(17)	15(17)
			16(14)	35(12)**	25(14)	14(14)
	Red 2G	10	15(14)	13(16)	NS	NS
			17(17)	17(17)		
			16(16)	13(14)		
	Brown FK	10	22(14)	47(15)**	S**	NS
			16(12)	43(12)**		
			17(14)	41(14)**		
		1	14(15)	39(15)**	S**	NT
			16(16)	35(16)**		
			14(12)	33(12)**		

NS = Not significant S = Significant NT = Not tested

†Cells were preincubated with caecal extract only or preincubated with caecal extract followed by microsomal activation.

‡The results not given in detail are detailed in Haveland-Smith & Combes, 1980.

Values marked with asterisks differ significantly from the control values (*$P < 0.05$; **$P < 0.01$).

whole-cell samples of *Fusobacterium* sp. 2, a human intestinal bacterium, are capable of reducing azo dyes (Chung, Fulk & Egan, 1975). Cell-free extracts of this bacterium reduced trypan blue to a mutagen in the Ames test (Hartman, Fulk & Andrews, 1978). *Bacteroides fragilis* subsp. *thetaiotaomicron* and *Fusobacterium* sp. 2 isolates can reduce methyl orange (*N,N*-dimethylaminoazobenzene-4-sulphonic acid) and activate this dye in an Ames test. This activity was attributed to the reduction product *N,N*-dimethyl-*p*-phenylenediamine which was isolated from the culture filtrate (Chung *et al.* 1975).

Our data show that Red 2G with microsomes only was genotoxic to *E. coli* despite the fact that the dye was not susceptible to reduction by the tester strains. Although metabolism *in vivo* mediated by hepatic azo reductase may proceed, it is unlikely that any significant reduction of the dye would occur during aerobic incubation with microsomal extracts (Hernandez, Gillette & Mazel, 1967). Furthermore acetylation of the 8-amino group (Fig. 1) is thought to increase susceptibility to reduction (Walker & Ryan, 1971) and the non-acetylated dye Red 10B is also genotoxic. There is also other evidence suggesting that microbial reduction is more significant than microsomal metabolism (Nambara & Yamaha, 1975). However, Brown FK parent dye was reduced more readily during incubation with the tester bacteria and was genotoxic. This suggests that, unlike Brown FK, trypan blue, methyl orange and some other azo dyes (Garner & Nutman, 1977), an intact azo bond is necessary for Red 2G genotoxicity. Indirect evidence for this is provided by the results of testing Black PN. This azo dye, which was completely reduced by the tester strain (Table 4), was non-genotoxic (Haveland-Smith & Combes, 1979) despite the fact that one of the expected reduction products (8-acetamido-2-amino-1-hydroxy-naphthalene-3,5-disulphonic acid) differs only in the substitution position of a sulphonic acid group from the naphthalene derivative which could arise from Red 2G. A 2-hr preincubation period with caecal extract produced a roughly 30-fold increase in the Cs

Table 4. *Decolourization of dyes*

Dye	Decrease in absorbancy at λ max. (%) after incubation with: *S. typhimurium* strain TA1538	*S. typhimurium* strain TA1535	*E. coli* WP2 *uvr A*	Caecal extract
Brown FK	24	NT	27	57
Red 2G	0	2	4	53
Black PN	100	NT	100	99

NT = Not tested

values for activated Red 2G (Table 2). Furthermore, since 24-hr incubation of Red 2G with these extracts caused a 53% decrease in coloration (Table 4), the 2-hr preincubation is likely to result in only a relatively small decrease in coloration. Hence, it would appear that the indirect activity of Red 2G is extremely sensitive to such preincubation.

Further evidence that reduction of Red 2G is inactivating arises from the observation that reduction mediated by dithionite did not activate Red 2G or Red 6B and reduction mediated by rat caecal microflora did not activate Red 2G. The direct activity of Red 10B was negated by chemical reduction. Furthermore, previously reduced Red 2G was not activated by microsomes. These results, together with the fact that Red 2G may be subject to significant microbial reduction *in vivo*, imply that this food colour is predominantly detoxified after ingestion. Brown FK, on the other hand, may be activated after intestinal reduction. To verify this, Brown FK was similarly incubated with caecal microorganisms prior to testing. The results show that this pretreatment did not reduce the indirect activities of the dye in the microsomal rec and fluctuation assays. Mutagenicity of the colouring with one tester strain was slightly potentiated.

Nevertheless, caecal extract did reduce the direct activity of Brown FK in the fluctuation test and resulted in more than twice the amount of decolourization of the dye than brought about by the tester strains. This may be explained if direct activity requires intact azo bonds. The extra amount of reduction caused by caecal extract may not have been sufficient to enhance greatly the indirect activity of the dye but was enough to reduce its direct effects. The lack of direct activity of Brown FK and the apparent inability of caecal pretreatment alone to activate the dye in the repair test may be due to the fact that this assay is less sensitive than fluctuation.

The foregoing discussion concerning the activities of the red dyes assumes that they are themselves the active ingredients of the samples tested. This assumption has been made on the basis of indirect evidence despite the observation of a small amount of a differently coloured impurity in the Red 2G sample used. Firstly Red 10B, which was also active, did not possess any impurity detectable on chromatographs. Secondly, loss in activity of the dyes due to reduction was accompanied by a decline in absorbancy measured at the λ max. value for each dye. Further analyses of these dyes and investigations into their genotoxicities using different batches are in progress.

After oral ingestion of Red 2G by rabbits the reduction products aniline (as *p*-aminophenol) and 8-acetamido-2-amino-1-hydroxynaphthalene-3,6-disulphonic acid are excreted (Daniel, 1962). There is therefore evidence for significant *in vivo* reduction of this dye. If the molecule was to remain intact it would be expected to be excreted rapidly due to the sulphonic acid groups. However, these polar groups are only present on one side of the azo link on the naphthalene moiety, the amine reduction product of which has not been tested for mutagenicity. The other reduction product of Red 2G, aniline, has been shown to be inactive with or without S9 mix (Garner & Nutman, 1977; McCann *et al.* 1975). However, it is active when tested in the presence of norharman and microsomes *in vitro* (see Ashby & Styles, 1978). It is possible that norharman competes with aniline for a *C*-hydroxylation pathway. Aniline, itself a non-carcinogen (Hartwell, 1951), is then preferentially *N*-hydroxylated forming, instead of *p*-aminophenol, phenylhydroxylamine which despite being non-carcinogenic (Miller & Miller, 1969) may be mutagenic (Serban, Duca-Marinescue, Museteanu & Maximilian, 1973). Thus, although our data imply that reduction of Red 2G is deactivating, it is feasible that potentially hazardous hydroxylamines could arise *in vivo* from aniline if the patterns of hydroxylation were altered, perhaps by the presence of a chemical in the diet acting like norharman.

It is interesting to note that, in addition to Red 2G, Brown FK, also found to be genotoxic, is similarly reduced to unsulphonated moieties, a property considered undesirable for a food colour (Cramer, Ford & Hall, 1978; Nurston, 1973).

The data presented suggest that the two dyes Red 10B and Red 6B, structurally-related to the indirect acting mutagen Red 2G, are respectively direct-acting and inactive in bacterial genetic systems. By comparing the structures of these three colourings it is suggested that one possible route for Red 2G activation may involve deacetylation. Since this is not a reaction thought to occur *in vivo* and since chemical and microbial reduction abolish the activity of Red 2G, a dye expected to be reduced *in vivo*, it is likely that this food colour may be deactivated after ingestion. The indirect activity of Brown FK is not lost after reduction and this food colour may therefore be more of a hazard.

The problems in extrapolating from the results of such short-term tests in order to assess hazard cannot be overstressed. The activities of these food colours in other genetic systems and their modes of activation are being studied further.

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SPECIES DIFFERENCES IN THE HAEMORRHAGIC RESPONSE TO BUTYLATED HYDROXYTOLUENE

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Abstract—Butylated hydroxytoluene (BHT) was administered in the diet to Sprague–Dawley, Wistar, Donryu and Fischer rats, ICR, ddY, DBA/c, C3H/He, BALB/cAn and C57BL/6 mice, New Zealand White-Sat rabbits, beagle dogs and Japanese quail, and by ip injection to Hartley guinea-pigs and Syrian golden hamsters. Haemorrhagic death occurred and the prothrombin index was decreased in all strains of rats given 1·2% BHT in the diet. Intra-epididymal and subcutaneous haemorrhages were observed in ddY mice and the prothrombin index was significantly decreased in ddY, BALB/cAn and C57BL/6 mice that were fed 1·2% BHT. Cerebral haemorrhages were found in some guinea-pigs injected ip with 380 mg BHT/kg/day for 3 successive days. Haemorrhages were not observed in hamsters, rabbits, dogs and quail treated with BHT. 2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone was detected in the livers of all strains of rats, but BHT was present in the form of non-conjugated phenols in the livers of the other species. 2,4,6-Tri-*tert*-butylphenol induced haemorrhagic death in rats, mice and hamsters but not in dogs. These results suggest that there is a species difference in the haemorrhagic effects of BHT.

INTRODUCTION

Butylated hydroxytoluene (BHT), a hindered phenolic antioxidant, can cause haemorrhagic death and a dose-dependent decrease in the prothrombin index in male rats when administered in the diet (Takahashi & Hiraga, 1978a,b). Vitamin K deficiency produced by a direct effect of BHT and/or its metabolites in the liver may be a central factor in this bleeding (Takahashi & Hiraga, 1979a). The nature of this effect, which differs from that of warfarin, has not yet been determined, but may be attributable to the structural characteristics of BHT rather than to its antioxidant properties (Takahashi & Hiraga, 1978c).

All of the data described above were obtained from experiments using only the male Sprague–Dawley rat as the experimental animal. A study of species differences is necessary as a basis for assessing the toxic hazard to man. The present report deals with the study of the haemorrhagic effect of BHT in various species. We also investigated whether or not 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, which was a major hepatic metabolite in the rat (Takahashi & Hiraga, 1979b), existed in the liver of various species.

EXPERIMENTAL

Materials. BHT was purchased from Tokyo Kasei Kogyo Co., 2,4,6-tri-*tert*-butylphenol from Aldrich Chemical Co., Milwaukee, WI, USA, and warfarin from Sigma Chemical Co., St. Louis, MO, USA. 2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone was synthesized by oxidation of BHT with lead dioxide (Filar & Winstein, 1960) and eluted with *n*-hexane from a silica-gel column (Takahashi & Hiraga, 1979b).

Diets. Before the experiments were begun the animals were given standard diets as described below. Vitamin K was not added to any of the diets. The crude protein contents of CLEA CE-2 and CLEA CG-3 and of CLEA dog food (CLEA Japan, Inc., Tokyo) were 24·5, 19·3 and 20·6%, respectively, and the crude protein contents of Funabashi RM-1 (Funabashi Farms, Chiba) and Udzura (Nisshin Flour Milling Co., Tokyo) were 17·3 and 23·0% respectively. In the feeding tests on all the animals except rabbits, dogs and quail, BHT and TBP were incorporated into a purified basal diet that contained 24% casein and was made up as described previously (Takahashi & Hiraga, 1978a). The chemicals were incorporated into the standard diets for rabbits, dogs and quail. Hamster and guinea-pigs were given the standard diets during the ip-injection tests.

Animals. Male and female rats, 4-wk-old, of the following strains were used: Sprague–Dawley (CLEA Japan Inc.), Wistar (CLEA Japan Inc.), Donryu (Nihon Rat Co., Saitama) and Fischer (F344/Du; Charles River Japan Inc., Kanagawa). The following strains of 4-wk-old male mice were used: ICR (CLEA Japan Inc.), ddY (Shidzuoka Agricultural Cooperative Association for Laboratory Animals, Shidzuoka), and DBA/2, BALB/cAn, C3H/He and C57BL/6 all of which were obtained from Charles River Japan Inc. Both the mice and the rats were fed a standard diet of CLEA CE-2 for 2 wk before they were used in BHT-feeding experiments. Male Syrian golden hamsters (Saitama Laboratory Animals Supplier, Saitama), 15–16 wk old, were used in experiments involving the ip injection of BHT, and 6-wk-old male and female hamsters (Murai Laboratory Animals, Saitama) were used in the TBP-feeding test. Both sets of hamsters were obtained when 5 wk old and were fed CLEA CE-2 until they were used in the experiments. Male Hartley guinea-pigs (mean body weight *c.* 200 g; Saitama Laboratory Animals Supplier) were fed a standard ration of CLEA CG-3 until they weighed 300 g, and then they were given

BHT. Male New Zealand White-Sat rabbits were obtained from the Nippon Bio-Supply Center, Tokyo at 2 months of age and were fed Funabashi RM-1 for 2–3 months before they were used in the BHT feeding tests. All the experiments using rats, mice, golden hamsters, guinea-pigs and rabbits were carried out at 25 ± 1°C and 50 ± 5% relative humidity with an 11/13 hr light/dark cycle. Male beagle dogs were obtained from Nihon E.D.M. Co., Shidzuoka at 6 months of age and were fed a standard diet of CLEA dog food for 4 months and then treated with BHT. The experiments using dogs were carried out at 23 ± 1°C and 50 ± 5% relative humidity with a 13/11 hr light/dark cycle. Male Japanese quail (white egged strain; Tokyo Metropolitan Livestock Experimental Station) were obtained when they were 56 days old, and were fed a standard diet of Uddzzura for 1 wk before they were given BHT.

Test procedures. Mice and rats were caged individually and given feed containing 1·2% BHT for 1 and 3 wk, respectively. Japanese quail were given 1% BHT in the diet for 17 days. Rabbits were fed BHT at a level estimated to provide 177 or 760 mg BHT/kg body weight/day for 2 wk. Dogs were given BHT in the diet at a level estimated to provide 173, 440 or 760 mg/kg body weight/day for 2 wk. [In an earlier study (Takahashi & Hiraga, 1978a), 760 mg/kg/day was determined to be the $LD_{50(40\ days)}$ for male Sprague–Dawley rats.] In each feeding experiment a control group was given untreated feed. For the study of the effects of BHT on dogs, male Sprague–Dawley rats were used as positive controls and were fed CLEA dog food containing 1·2% BHT (mean intake = 876 mg/kg body weight/day) for 10 days.

BHT was administered ip to hamsters at a level of 380 or 760 mg/kg body weight/day for 3 days, and to guinea-pigs at 190 or 380 mg/kg body weight/day for 3 days. Male Sprague–Dawley rats were used as a positive control group and were given 380 or 760 mg BHT/kg body weight/day ip for 3 days. In all cases the BHT was administered in solution with acetone–soya bean oil (1:9, v/v; 200 mg BHT/ml). Control animals were injected with 3·8 ml acetone–soya bean oil/kg. TBP was administered at a level of 1·43% in the diet to male Sprague–Dawley rats or to male hamsters for 3 or 2 wk, respectively. Male ddY mice were fed 0·57% TBP for 10 days and male ICR mice were given 0·57 or 1·43% TBP in the diet for 10 days. TBP was fed to dogs at a level estimated to provide 49·2, 173 or 519 mg/kg body weight/day for 16 days. During the first two days of dosing decreased body temperature, ptosis, increased salivation and grogginess were observed in dogs given the highest dose and so for the remaining 14 days the dose was reduced to 439 mg/kg/day.

Male ICR mice and beagle dogs were given warfarin in the diet at levels of 0·002% and 1·33 mg/kg weight/day, respectively, for 3 days as a positive control for haemorrhagic effects.

The numbers of deaths and of external haemorrhages, general signs of intoxication, body weights and food intakes were recorded throughout the experiments. The rats, mice, hamsters, guinea-pigs and quail were killed and the numbers of internal haemorrhages were recorded and prothrombin times and liver weights were measured. Prothrombin time was estimated by the one-stage method of Quick, Stanley-Brown & Bancroft (1935). The prothrombin index was derived by a formula described previously (Takahashi & Hiraga, 1978a). It was not possible to determine the prothrombin time for quail using rabbit-brain thromboplastin, and instead the prothrombin concentration was determined by the method of Owren (1949). In the experiments using rabbits and dogs, plasma was collected successively and prothrombin times or partial thromboplastin times were determined. Serum glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and alkaline phosphatase activities were determined.

The effects of feeding 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone or BHT on the concentrations of factors II and VII in the plasma of male Sprague–Dawley rats were determined. The rats were starved for 24 hr and then given feed containing 80 mg of the quinone methide or BHT each day for two days. BHT was stable, but the corresponding quinone methide content of the feed decreased to 55% after 24 hr. The mean daily intake per rat of BHT or the quinone methide was calculated to be 53 or 80 mg, respectively. Plasma from the treated animals was diluted tenfold and added to plasma lacking factors II or VII and a prothrombin time assay was performed. The concentration of each factor in the plasma of the treated rats was estimated from a plot of factor concentrations against prothrombin times for normal rat plasma.

Tissue analyses. The livers of rats, mice, hamsters, guinea-pigs, rabbits and quail that had been given BHT were homogenized with acetone and the unconjugated phenols were extracted. 2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone was determined by gas chromatography. The detailed procedures for tissue analysis were as previously described (Takahashi & Hiraga, 1979b).

Statistical methods. The results were analysed by Student's *t* test and the minimum level of statistical significance accepted was $P < 0·05$ (Snedecor & Cochran, 1967). Median lethal time (Lt_{50}) was estimated by the method of Litchfield (1949).

RESULTS

Haemorrhagic effects of BHT

Haemorrhagic deaths occurred among male rats of all strains and female rats of the Fischer strain that were given BHT (Table 1). Haemorrhages were observed in all the rats except in females of the Donryu strain. No obvious internal haemorrhages were observed in female Sprague–Dawley rats, but in the other rats haemorrhages were found in the muscle, testis, epididymis, nasal cavity and cranial cavity, and haemorrhagic anaemia was also observed. In male Sprague–Dawley rats, massive haemorrhages were observed in the pleural cavity and these were coincident with a high death-rate in this strain (Fig. 1). In earlier studies a high incidence of peritoneal haemorrhages has been observed in Sprague–Dawley rats given BHT (Takahashi & Hiraga, 1978a,c & 1979a). The prothrombin index was significantly decreased in all strains of rats except in female Donryu rats (Table 1). In ddY mice given BHT in the diet, intra-epididymal and subcutaneous haemorrhages were observed. The

Table 1. *Haemorrhagic effects of BHT in different species*

Species and strain	Sex	Dose of BHT†	Mean intake of BHT (mg/kg/day)‡	Percentage of total population with haemorrhages		Mean prothrombin index (%)	Hepatic level of quinone methides§ (μg/g liver)
				Dead	Surviving		
Rat							
Sprague–Dawley	M	0	0 (31)	0	0	101	
		1·2%	693 (50)	44	50	18***	NC
	F	0	0 (5)	0	0	101	
		1·2%	1000 (10)	0	30	73***	NC
Wistar	M	0	0 (5)	0	0	100	
		1·2%	638 (10)	10	90	22***	38
	F	0	0 (5)	0	0	100	
		1·2%	854 (10)	0	100	38***	7
Donryu	M	0	0 (5)	0	0	100	
		1·2%	1120 (10)	10	70	13***	41
	F	0	0 (5)	0	0	100	
		1·2%	1000 (10)	0	0	92	27
Fischer	M	0	0 (5)	0	0	100	
		1·2%	821 (10)	30	70	5***	16
	F	0	0 (5)	0	0	100	
		1·2%	895 (10)	20	70	18***	11
Mouse							
ddY	M	0	0 (5)	0	0	100	
		1·2%	1701 (10)	0	30	79**	ND
ICR	M	0	0 (10)	0	0	100	
		1·2%	1344 (10)	0	0	96	ND
DBA/2	M	0	0 (10)	0	0	100	
		1·2%	847 (10)	0	0	138**	NC
BALB/cAn	M	0	0 (5)	0	0	100	
		1·2%	1730 (10)	0	0	84**	NC
C3H/He	M	0	0 (10)	0	0	100	
		1·2%	1858 (10)	0	0	115***	NC
C57BL/6	M	0	0 (5)	0	0	100	
		1·2%	1925 (10)	0	0	91*	NC
Hamster							
Syrian golden	M	0	0 (5)	0	0	101	
		380 mg/kg	380 (4)	0	0	101	
		760 mg/kg	760 (6)	0	0	87	ND‖
Guinea-pig							
Hartley	M	0	0 (5)	0	0	100	
		190 mg/kg	190 (5)	0	0	78	
		380 mg/kg	380 (5)	0	0	73	ND
Japanese quail							
White egged	M	0	0 (5)	0	0	100	
		1%	1056 (5)	0	0	53*	ND

BHT = Butylated hydroxytoluene NC = Not calculated ND = Not detected

†Expressed as percentage in the diet or mg/kg body weight administered ip.

‡The numbers in brackets are the numbers of animals in each group.

§2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone.

‖Quinone methide levels were determined in separate experiments in which BHT was administered to hamsters or guinea-pigs at 1·2 or 1·0% in the diet, respectively, for 3 days.

The values marked with asterisks differ significantly (Student's *t*-test) from the corresponding control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$. The times of treatment for rats, mice, hamsters, guinea-pigs and quail were 3 wk, 1 wk, 3 days, 3 days and 17 days, respectively.

prothrombin index was significantly decreased in ddY, BALB/cAn and C57BL/6 strains of mice fed BHT, but was significantly increased in the DBA/2 and C3H/He strains (Table 1). The prothrombin index of male ICR mice given 0·002% warfarin in the diet was decreased significantly ($P < 0·001$) to only 6% of the control value.

BHT injected ip at either 380 or 760 mg/kg did not significantly decrease the prothrombin index of hamsters (Table 1), but the same ip doses of BHT significantly decreased the prothrombin index of male Sprague–Dawley rats to 69% ($P < 0·05$) or 46% ($P < 0·001$) of the control value, respectively. Cerebral haemorrhages and tremor were observed in two guinea-pigs given 380 mg BHT/kg ip. In the groups of guinea-pigs given 190 or 380 mg BHT/kg the prothrombin index was decreased to 78 or 73% of the control values, respectively (Table 1). The prothrombin time of guinea-pigs, determined using rabbit-brain thromboplastin, was relatively longer (86 sec) than that of other species (10–30 sec), other than the quail. The lack of significance ($P = 0·06$) in the difference

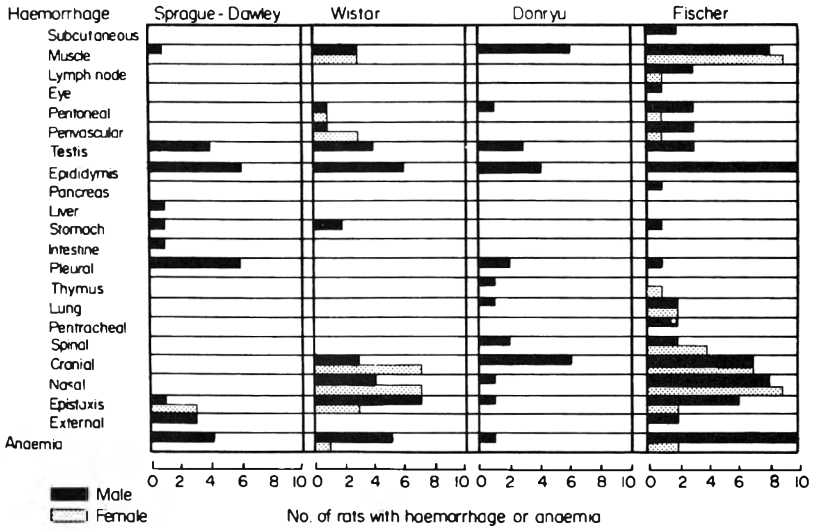

Fig. 1. Numbers of male (■) and female (▨) rats of four different strains observed with haemorrhages or anaemia after being fed on a diet containing 1·2% butylated hydroxytoluene for 3 wk.

between the prothrombin index of the controls and of the guinea-pig groups treated with BHT may be attributable to the wide variations in prothrombin indices between the animals in each group.

In male rabbits fed BHT at either dose level for 2 wk the prothrombin index did not decrease (Table 2) and no haemorrhages were observed. Neither were haemorrhages observed in dogs given BHT. The prothrombin index was not decreased in dogs fed BHT (Table 3), but it decreased significantly ($P < 0·01$) to 8% of the control value in dogs given warfarin and to 29% ($P < 0·01$) of the control value in male Sprague–Dawley rats given CLEA dog food containing 1·2% BHT. In quail fed BHT, no haemorrhages were observed but the prothrombin concentration was significantly decreased (Table 1).

The quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, was detected in the livers of both sexes of Wistar, Donryu and Fischer rats in this study and was also found to be a hepatic metabolite of BHT in Sprague–Dawley rats in an earlier study (Takahashi & Hiraga, 1979b). Quinone methide was not detected in the livers of the other species; in these BHT was present in the form of non-conjugated phenols.

Haemorrhagic effects of TBP

All the rats given TBP died with haemorrhages (Table 4). Haemorrhagic effects of TBP in male Sprague–Dawley rats were also reported in an earlier study (Takahashi & Hiraga, 1978c). In the female Sprague–Dawley rats haemorrhages were observed in the pleural, peritoneal, cranial and nasal cavities, and in the muscle, thymus, pancreas, spinal column and bladder wall. Intragastric blood pooling also occurred. The Lt_{50} and its slope function in female rats were 12·5 days (10·6–14·8 days; 95% confidence limits) and 1·3 (1·2–1·5), respectively. The time–lethal effect curve for female rats was parallel to that for the males. There was a significant difference between the Lt_{50} of the females in this study and that of 7·4 days reported for male rats in an earlier study (Takahashi & Hiraga, 1978c). Haemorrhages were not observed in either strain of mice that were given 0·57% TBP in the diet but ICR mice that were given the higher level of TBP (1·43%) died with haemorrhages (Table 4). Haemorrhages were observed in the pleural, peritoneal, cranial and nasal cavities and in the muscle, lungs, thymus, liver, gall bladder, intestine, anus, epididymis, spinal column and bladder wall. The Lt_{50} and its slope function for ICR mice given the higher

Table 2. *Prothrombin index of male rabbits fed BHT for 2 wk*

Rabbit no.	BHT intake (mg/kg/day)	Prothrombin index before BHT administration	Prothrombin index (%) following BHT administration for	
			1 wk	2 wk
1	177	101	96	91
2	177	96	115	108
3	242*	101	115	96
4	390*	101	115	108

BHT = Butylated hydroxytoluene

*Food consumption was decreased in rabbits given the higher dose of BHT.

Table 3. *Prothrombin index and partial thromboplastin time of male dogs fed BHT for 2 wk*

Dog no.	BHT intake (mg/kg/day)	Prothrombin index before BHT administration (%)	Prothrombin index (%) after BHT administration for 1 wk	Prothrombin index (%) after BHT administration for 2 wk	Partial thromboplastic time (sec) after BHT administration for 1 wk	Partial thromboplastic time (sec) after BHT administration for 2 wk
1	0	100	84	111	54	34
2	0	100	84	131	39	43
3	173	102	107	150	39	37
4	173	98	90	141	49	45
5	440	111	131	117	35	42
6	440	91	68	105	37	39
7	760	93	120	137	59	37
8	760	108	158	137	33	39

BHT = Butylated hydroxytoluene

dose of TBP were 6·8 (5·7–8·2) days and 1·3 (1·2–1·5), respectively. The time–lethal effect curve of the male ICR mice was parallel to that of the male rats and the Lt_{50}s of the male rats and mice were not significantly different. The administration of TBP significantly decreased the prothrombin index in male mice. Haemorrhages were observed in both male and female hamsters fed TBP, but haemorrhages were also found in control animals. A massive external haemorrhage of the rectum was observed in one male hamster that died following TBP administration. TBP significantly decreased the prothrombin index in both male and female hamsters (Table 4). No haemorrhages were observed, and there was no decrease in either the prothrombin index or the partial thromboplastin time in dogs given TBP (Table 5). Increases in the activities of serum glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and alkaline phosphatase and a depression of the central nervous system were observed in dogs that were fed TBP.

DISCUSSION

There were wide variations in the haemorrhagic effects of BHT in the different species. Rats were generally susceptible to the haemorrhagic effects of BHT, although those of the Donryu strain were more resistant than the other strains tested. Female rats were more resistant to the haemorrhagic effects of BHT than were the male rats. Guinea-pigs and ddY mice showed some haemorrhagic response to BHT administration. No haemorrhages were induced in the other species tested, although following BHT administration significant reductions in prothrombin levels were observed in ddY, BALB/cAn and C57BL/6 mice and in Japanese quail. The effects of BHT on mice were very different from those on dogs, which were resistant to the haemorrhagic effects of both BHT and TBP. Hamsters were more resistant to TBP than were rats.

The animals used in our experiments possess similar coagulation systems with the exception of the

Table 4. *Haemorrhagic effects of 2,4,6-tri-tert-butylphenol (TBP) in rats, mice and hamsters*

Species and strain	Sex	Dose of TBP (% in diet)	TBP intake (mg/kg/day)†	Percentage of total population with haemorrhages: Dead	Percentage of total population with haemorrhages: Surviving	Prothrombin index (%)
Rat						
Sprague–Dawley	M	0	0 (6)	0	0	—
		1·43	519 (10)	100	0	<2‡
	F	0	0 (5)	0	0	—
		1·43	227 (10)	100	0	—
Mouse						
ddY	M	0	0 (6)	0	0	100
		0·57	NC (10)	0	0	37**
ICR	M	0	0 (5)	0	0	100
		0·57	NC (10)	0	0	52**
		0	0 (5)	0	0	—
		1·43	1275 (10)	100	0	—
Hamster						
Syrian golden	M	0	0 (4)	25	25	100
		1·43	1092 (10)	10	30	38*
	F	0	0 (5)	20	0	100
		1·43	801 (10)	10	0	52*

NC = Not calculated

†The numbers in brackets are the numbers of animals in each group.

‡The prothrombin index was determined in a separate experiment in which male Sprague Dawley rats were given 1·43% TBP in the diet for 5 days.

The values marked with asterisks differ significantly from the corresponding control values: $*P < 0{\cdot}01$; $**P < 0{\cdot}001$. Rats, hamsters and mice were fed TBP for 3 wk, 2 wk and 10 days, respectively.

Table 5. *Prothrombin index and partial thromboplastin time of male beagle dogs fed 2,4,6-tri-tert-butylphenol (TBP) for 16 days*

Dog no.	TBP intake (mg/kg/day)	Prothrombin index (%) on day*						Partial thromboplastin time (sec) on day*	
		−26	−19	0	+3	+7	+16	+7	+16
1	0	120	85	103	104	102	89	45	50
2	0	86	122	98	96	98	115	51	42
3	49	93	92	103	78	96	96	44	58
4	49	108	110	130	79	129	116	55	43
5	173	91	102	108	133	114	73	39	36
6	173	111	98	103	73	96	134	36	31
7	449	99	91	139	93	94	79	31	31
8	449	101	111	51	109	115	113	37	31

*Negative values indicate the number of days before TBP administration began. The prothrombin index was determined 7 hr after TBP feeding was started (Day 0). Positive figures indicate the number of days after TBP feeding was started.

quail, which, in common with other birds, is deficient in several intrinsic factors (Bennett & Ratnoff, 1973; Didisheim, Hattori & Lewis, 1959; Kowarzyk, Czerwinska-Kossobudzka & Jacobi, 1971; Saito, Goldsmith & Ratnoff, 1974; Walz, Kipfer & Olson, 1975). However, differences do exist between the coagulation systems of mammalian species. For example, the extrinsic coagulation system of the guinea-pig is relatively weak, as is evident from the relatively longer prothrombin time observed in these studies; the coagulation factors V, VIII, XI and XII of rats as well as the prothrombin-complex are decreased in rats by warfarin treatment (Owen & Bowie, 1978), the hamster is relatively resistant to warfarin (Shah & Suttie, 1975), and the content of hepatic vitamin K varies between different species, strains and sexes (Duello & Matschiner, 1970, 1971 & 1972; Knauer, Siegfried & Matschiner, 1976). These differences in coagulation systems may modify the effect of BHT. However, variation between species in their metabolism of BHT is probably the most important factor that determines the differences in haemorrhagic response to the antioxidant. 2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone has been reported to be a major hepatic metabolite of BHT in rats (Takahashi & Hiraga, 1979b) and was detected in the livers of rats of both sexes in this study. However this compound was not detected in the livers of any of the other species tested. The concentrations of factor II (prothrombin) and factor VII rapidly decreased in plasma taken from rats given 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone in the diet for 2 days (Table 6). However the level of this quinone methide in the livers of rats fed 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone was lower than in the livers of rats given BHT. Therefore, it is evident that the haemorrhagic effect of this quinone methide should be studied by another method. Detailed studies of the haemorrhagic effects of BHT using guinea-pig or ddY mice, the livers of which contain BHT in the form of non-conjugated phenols and not the corresponding quinone methide, are also necessary in order to understand whether or not the haemorrhagic mechanisms in these species are similar to those in the rat.

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Table 6. *Effects of feeding 2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone or BHT on the concentrations of factors II and VII in the plasma of male Sprague–Dawley rats*

Treatment	No. of rats	Daily chemical intake (mg/rat)	Concentration in plasma (%) of		Hepatic level of quinone methide† (μg/g liver)
			Factor II	Factor VII	
Control	5	0	100	100	0
Quinone methide	5	44–80‡	70*	35**	1·2
BHT	5	53§	88	111	11·6

BHT = Butylated hydroxytoluene

†2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone.

‡Rats were given feed containing 80 mg quinone methide each day for 2 days. Quinone methide was unstable and decreased to 55% of the original level over 24 hr and therefore the daily intake of quinone methide by the rats was within the range 44–80 mg.

§Food consumption was decreased in rats given feed containing 80 mg BHT each day for 2 days.

The values marked with asterisks differed significantly from the corresponding control values: *$P < 0.01$; **$P < 0.001$.

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EVALUATION OF THE MUTAGENICITY OF SORBIC ACID–SODIUM NITRITE REACTION PRODUCTS PRODUCED IN BACON-CURING BRINES

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Abstract—Typical bacon-curing brines containing potassium sorbate (2600 ppm), sodium nitrite (40–4000 ppm) and sodium ascorbate (550–55,000 ppm) were reacted under conditions simulating gastric fluid or the curing or cooking of bacon. Ames Salmonella assays were conducted on the reaction solutions as well as on their ether extracts. All sorbate–nitrite solutions and most ether extracts were negative in this assay. A positive response was detected in the high-temperature sample, but retesting of this sample indicated that the positive response was due to an active compound in the ether used as the extraction solvent.

INTRODUCTION

Several reports have been published in the past 5 yr about the formation of mutagenic or DNA-damaging reaction products when high concentrations of sorbic acid and sodium nitrite (130,000 ppm) react under strongly acidic conditions (Hayatsu, Chung, Kada & Nakajima, 1975; Kada, 1974 & 1976; Namiki & Kada, 1975). At 0°C a nitrite-labile, red oily extract resulted (Hayatsu *et al.* 1975). This was positive in the *Escherichia coli* Sd-B (TC) assay, indicating DNA-damaging potential. Kada (1974) and Namiki & Kada (1975) reported reactions between 34,500 ppm nitrite and 2000 ppm sorbic acid at 90–100°C. The sorbate–nitrite combination had 'some positive results' in the *Bacillus subtilis* recombination assay but no numerical data were reported. Namiki & Kada (1975) reported that the sorbate–nitrite reaction mixture, when extracted with dichloromethane and ether, produced ethylnitrolic acid (ENA) with a 'marked potency' in the *B. subtilis* recombination assay.

Khoudokormoff (1978) found that mixtures of 100–400 ppm sodium nitrite plus 0·1% potassium sorbate were positive in the *B. subtilis* M45 recombination assay below pH 6·0 in an unbuffered medium. The same author reported negative results using the *B. subtilis* M45 assay at pH 6·0 or above. He also found that the sorbate–nitrite mixture was negative in the Ames Salmonella assay as well as in the *E. coli* WP2 and WP2*uvr*A$^-$ system regardless of the pH of the reaction mixture.

In another recent publication, Tanaka, Chung, Hayatsu & Kada (1978) reported that lower levels of nitrite (13,000 ppm) when reacted with sorbic acid (2000 ppm) at a variety of pH values (1–5) gave reaction products that were not positive in the Ames Salmonella assay or the *B. subtilis* recombination assay. Very little reaction took place between sorbate and nitrite at or above pH 5·0. Namiki, Udaka, Osawa, Tsuji, Namiki & Kada (1979) described reaction conditions and products of sorbic acid and sodium nitrite: the molar ratio of sorbic acid:sodium nitrite had to be at least 20:80 for a positive response in the *B. subtilis* rec-assay at pH 3·4 after the mixture had been heated at 60°C for 1 hr. Mixtures of sorbic acid and sodium nitrite (20:160 molar ratio) heated at 60°C for 1 hr gave positive responses in the *B. subtilis* rec-assay below pH 5 but were negative above that point. The same authors observed the formation of 1,4-dinitro-2-methylpyrrole in the Namiki & Kada (1975) reaction. This compound was positive in the Salmonella assay but the addition of ascorbic acid to the mixture eliminated the mutagenicity. These authors also noted that the addition of ascorbic acid to a sorbic acid–sodium nitrite reaction mixture prevented the development of mutagenic activity.

Many of these experiments were conducted under extreme conditions and there is a need for the potential for forming mutagenic products to be assessed under realistic conditions. Interpretation of the significance of these results is confounded by the facts that nitrite itself is mutagenic, that the mutagenic potency of nitrite is pH sensitive, that olefin–nitrite adducts are nitrite labile, and that ascorbate will trap free nitrite under the appropriate conditions.

The work reported here is a study of the potential formation of mutagenic reaction products when potassium sorbate and sodium nitrite are present together in bacon-curing brines, or in bacon under simulated commercial conditions. The potential formation of ENA and its thermal decomposition are also reported.

EXPERIMENTAL

Materials. Potassium sorbate and sodium tripolyphosphate were food-grade commerical products of Monsanto Company, St. Louis, MO. Sodium nitrite and sodium chloride, reagent grade, were supplied by Fisher Scientific Co., St. Louis, and sodium ascorbate, reagent grade, was supplied by Sigma Chemical Company, St. Louis. The extraction solvent was technical-grade ether supplied by Mallinkrodt Co., St. Louis, or a purified sample obtained using the method described by Vogel (1966).

Reaction conditions. The reaction conditions used to prepare products for the first series of mutagenicity tests are shown in Table 1. The Hayatsu *et al.* (1975) reaction was carried out as a positive control, the extract being assayed to confirm that the method, as

Table 1. *Reaction variables for test 1*

Treatment	Concn of components of mixture				Reaction		
	Sorbate (ppm)	Nitrite (ppm)	NaCl %	Ascorbate (ppm)	pH	Time (hr)	Temperature (°C)
Hayatsu	25,000	130,000	0	0	<1·0	3	0
Bacon cure							
1	2600	40	1·5	550	7·0	8	55
2	2600	40	1·5	550	6·5	8	55
3	2600	4000	1·5	55,000	6·5	8	55
4	2600	40	1·5	550	6·5	8	0
5	2600	40	1·5	550	5·0	8	55
High-temp. reaction							
6	2600	40	1·5	550	6·5	8/0·1*	55/100*
7	0	40	1·5	550	6·5	8/0·1*	55/100*
Brine							
8	26,000	400	15	5500	7·2	12	25
Gastric fluid							
9	2600	40	1·5	550	1·0	3	37

*Reaction proceeded for 8 hr at 55°C followed by 0·1 hr at 100°C.

used in both the Monsanto Environmental Health Laboratory (EHL) and the Monsanto Research Corporation Laboratory (MRC), was adequate for detecting mutagenic products. The Hayatsu solution contained 1·12 g sorbic acid, 6·90 g sodium nitrite, 12 ml conc. HCl, 20 ml acetic acid and 20 ml H_2O, and was stored at 0°C for 3 hr at a pH below 1·0 prior to ether extraction and testing by the Salmonella assay. The other reactions outlined in Table 1 simulated conditions encountered during bacon curing, bacon cooking and brine storage, and in gastric fluid. In all these cases, the undiluted solution was assayed and, whenever possible, ether extracts were prepared and assayed.

Ether extractions. The mixture from the positive control (Hayatsu *et al.* 1975) was extracted with ether, the ether was evaporated, the residue was redissolved in ether, the solution was washed with water and the ether was removed. The final residue was tested by the Ames Salmonella assay at the two different Monsanto laboratories. Reaction mixtures with a higher pH were generally adjusted to pH 5·0 with conc. HCl. Ether extracts of the solutions resulting from treatments 1–5, 7 and 8 were prepared by extracting the reaction mixture (750 ml) with 10 × 100 ml diethyl ether. After each extraction, the pH of the aqueous solution was readjusted to pH 5·0. The pooled extracts were washed twice with 20 ml distilled water, dried over 50 g anhydrous Na_2SO_4, filtered and concentrated on a rotary evaporator (<50°C) to dryness. The ether extract of the treatment-6 solution was prepared in the same way, except that one 50-ml water-wash was used, the pH was not adjusted (it remained acidic) and anhydrous $MgSO_4$ was used as the drying agent. In treatment 9 (gastric fluid), the solution was extracted with 5 × 200 ml ether, and the pH was not adjusted. In a second series of tests treatments 10–13), undertaken to investigate further the results obtained with treatment 6 in the first series, ether extracts were prepared from the reaction mixtures as before, but using in some cases the commercial solvent and in the others a purified sample. All four mixtures were reacted for 16 hr at 55°C and 6 min at 100°C, and each contained 1·5% NaCl and 550 ppm ascorbate and had a pH of 6·5. Two (treatments 10 and 12) contained 2600 ppm sorbate and 40 ppm nitrite, while one (treatment 11) contained 40 ppm nitrite and no sorbate and the fourth (treatment 13) contained 2600 ppm sorbate and no nitrite. The mixtures from treatments 10 and 11 were extracted with purified ether and those from treatments 12 and 13 with the unpurified solvent. $MgSO_4$ was used as the drying agent for treatments 10–13.

Mutagenicity testing. Media and handling procedures were those reported by Ames, McCann & Yamasaki (1975). *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were all used to test the mutagenicity of the undiluted solutions resulting from all nine treatments, the ether extracts of these solutions (from treatments 1–5 and 7–9) and the Hayatsu reaction product, in the presence and absence of rat-liver S-9 fraction. In the second set of tests, assays were performed at EHL on the ether extracts only, using strain TA100 without microsomal activation.

Ethylnitrolic acid preparation, treatment and analysis. ENA, prepared according to the method of Namiki & Kada (1975), was dried and subjected to various high temperatures and then monitored for decomposition, gravimetrically and by infrared spectroscopy. Modifications of the Namiki & Kada (1975) method were implemented to determine the effect of varying levels of nitrite (125–1000 ppm) and of pH (3·4 and 6·0) on the formation of ENA in the presence of 2000 ppm sorbic acid. Paired-ion chromatography was used to analyse the undiluted reaction mixture, and adsorption chromatography to analyse the ether extract of the reaction mixture. Both analyses showed peaks with ENA retention times, confirming the compound's presence in the reaction mixture. A Waters Associates Liquid Chromatograph equipped with two M-6000 pumps, a model 660 solvent programmer and a Schoeffel Spectroflow SF770 Monitor were used for the separation. The columns were Waters Associates 30 cm × 4·6 mm (ID) μ Bondapak C_{18} (Cat. no. 27324) and μ Porasil (Cat. no. 27386).

For ion-chromatography analysis, the eluent for the

Table 2. *Results of Ames Salmonella assay on solutions and ether extracts resulting from treatments 1–9 (test 1)*

Treatment no. and sample	Laboratory*	Lowest toxic level (mg/plate)	Mutagenicity†
Hayatsu	EHL	0·04	pos. TA100
	MRC	0·10	pos. TA100
1 solution	MRC	>10	neg.
extract	MRC	1	neg.
2 solution	MRC	>10	neg.
extract	MRC	3	neg.
3 solution	MRC	>10	neg.
extract	MRC	3	neg.
4 solution	MRC	>10	neg.
extract	MRC	1	neg.
5 solution	MRC	>10	neg.
extract	MRC	10	neg.
6 solution	EHL	>10	neg.
extract	EHL	3	pos. TA100
7 solution	EHL	>10	neg.
control extract‡	EHL	0·67	pos. TA100
8 solution	MRC	>10	neg.
extract	MRC	10	neg.
9 solution	EHL	>10	neg.
extract	EHL	10	neg.

*Studies were carried out at either the Monsanto Environmental Health Laboratory (EHL) or the Monsanto Research Corporation Laboratory (MRC).
†Positive or negative tests results as indicated.
‡High-temperature treatment of nitrite solution (in absence of sorbate) followed by ether extraction.

first pump was distilled water containing 0·5 M-phosphoric acid and 0·015 M-tetrabutylammonium hydroxide, the pH being adjusted to 4·0 with sodium hydroxide. The second pump's eluent was distilled water–acetonitrile, 60:40 (v/v), containing 0·05 M-phosphoric acid and 0·015 M-tetrabutylammonium hydroxide, with pH adjustment to 4·0 with sodium hydroxide. The flow rate was 1·5 ml/min, at a pressure of 300 lb/in^2, and the detector was set at 242 nm. The eluent for adsorption chromatography was dichloromethane–hexane, 65:35 (v/v) at a flow rate of 2 ml/min.

RESULTS

The products of ether extraction of the Hayatsu *et al.* (1975) reaction mixtures were positive with strain TA100 in the Ames Salmonella assay (Table 2). All solutions and ether extracts and sorbate–nitrite samples produced under bacon-curing conditions (treatments 1–5) were negative in the Salmonella assay, as were the solution simulating the brine and its ether extract (treatment 8) and the simulated gastric-fluid sample (treatment 9) and its extract (Table 2). Weak mutagenic activity was detected in the ether extract of the high-temperature sample (treatment 6) although the solution itself was negative (Table 2). The ether extract control was also positive (Table 2) and its potency was sufficient to account for the results obtained with the high-temperature extract and with a sulphamate-treated high-temperature sample (Table 3).

In order to resolve questions regarding the mutagenicity of the high-temperature extract, additional

Table 3. *Comparison between ether extract control and the positive ether extracts derived from the high-temperature treatment (no. 6)*

Ether extract	Weight of sample (g)	Volume of ether* (litres)	Potency—slope (reversions/plate/mg) Observed	Potency—slope (reversions/plate/mg) Calculated as due to ether residue†
Control‡	0·066	1	$2·6 \times 10^3$	—
No. 6	1·3	1	$6·3 \times 10$	$1·3 \times 10^2$
No. 6 (sulphamate-treated extract)	0·24	2	$2·4 \times 10^2$	$1·4 \times 10^3$

*Total volume of ether used in extraction.
†Calculation: ether extract control potency/mg × 66 mg × volume of ether (litres) used for test extraction ÷ sample weight (mg).
‡Ether extract of nitrite solution subjected to high temperature in the absence of sorbate (treatment 7, Table 1).

Table 4. *Effect of pH and nitrite level on ENA formation in solution containing 2000 ppm sorbic acid*

pH	Concn of $NaNO_2$ (ppm)	ENA produced (ppm)
3·4	1000	38·8
3·4	500	22·4
3·4	250	1·7*
3·4	125	0·6†
6·0	500	−0·4

ENA = Ethylnitrolic acid
*Detection limit.
†Analytical zero.

limited studies were performed using strain TA100 without S-9 activation. The results demonstrated that none of the sorbate–nitrite mixtures treated at high temperatures were positive in the Salmonella assay. A small amount of activity presented in the ether extracts, including the control sample extracted with purified ether, may have been due to an active compound such as peroxide produced during the drying of the ether over $MgSO_4$ or Na_2SO_4.

Table 4 indicates that under acidic conditions ENA is not formed when less than 250 ppm nitrite is used in the reaction. It is not formed at all at pH 6·0, even when higher levels of nitrite (500 ppm) are used. These data suggested that ENA is not formed in cured meat or curing pickle. ENA is highly heat labile and decomposes in less than 1 sec at 170°C (cooking temperature for bacon). At 100, 70, 55 and 43°C, the times required for complete decomposition were 19 sec, 11·5 min and 2·0 and 17·5 hr, respectively. At 24°C, 9% decomposition occurred in 17 days. Infrared spectroscopy indicated that acetic acid and nitrogen oxides are the major decomposition products.

DISCUSSION

The basal level of sodium ascorbate in this study was set at 550 ppm to simulate the conditions of commercial bacon processing in the USA. The ratio of ascorbate to nitrite (ppm) was maintained at 550/40 when elevated concentrations of nitrite were used. This ratio conforms to the proposed Department of Agriculture regulation on the use of sorbate in bacon. The ability of ascorbate to react with nitrite is well documented. Mirvish, Wallcave, Eagen & Shubik (1972) reported ascorbate to be a blocking agent in nitrosamine formation. Williams (1978) described the effectiveness of ascorbate as a nitrite trap. Tanaka *et al.* (1978) demonstrated that ascorbic acid reacted rapidly with nitrite below pH 4 and was an effective nitrosamine inhibitor. Namiki *et al.* (1979) reported that ascorbic acid suppressed sorbic acid–sodium nitrite reactions and that the addition of ascorbic acid to a reaction mixture of sorbic acid and nitrite blocked the formation of compounds with mutagenic activity.

The results of the present study, performed under simulated commercial conditions, demonstrate that all sorbate–nitrite–ascorbate samples were negative in the Ames Salmonella assay. The only positive Ames results were attributed to the presence of mutagenic impurities in the ether used as the extraction solvent.

It is clear that under acidic conditions (pH 3·4), ENA is not formed at nitrite levels below 250 ppm, even in the absence of ascorbate, and is not formed at all at higher pH (6·0) even when higher levels of nitrite (500 ppm) are present. ENA could not be formed in cured meat or curing brine by any mechanism so far explored. In addition, ENA would not survive cooking temperatures, since it decomposes to acetic acid and nitrogen oxides in less than 1 sec at 170°C. Given the pH of cured meat and the presence of adequate ascorbate, the mutagenic 1,4-dinitro-2-methylpyrrole described by Namiki *et al.* (1979) would not form in a meat-curing situation. These authors pointed out that reaction conditions must include a low pH (3·5) and an excess of nitrite in order for the pyrrole to form. They also noted that the addition of ascorbic acid to the pyrrole prevented the expression of mutagenic activity.

In conclusion, one could expect a range of DNA-damaging and mutagenic responses with unstable ether extracts of sorbate–nitrite mixtures under conditions of low pH (≤ 1) and excess nitrite. The levels of sorbate and nitrite proposed for used in cured meats (0·26% sorbate and 40 ppm nitrite), however, together with a pH of at least 5·5 and the presence of ascorbate do not pose a hazard in regard to the formation of reaction products in cured meat or the curing brine.

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MUTAGENICITY OF CHINESE WINE TREATED WITH NITRITE

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Abstract—Mutagens were formed in seven out of 12 brands of Chinese wine tested after treatment with nitrite at pH 3. Most of the non-distilled wines were found to contain the precursors of mutagens while the distilled wines did not. The majority of Chinese wine is non-distilled and this, coupled with the over use of sodium nitrite in food processing in China, could be a cause of the high incidence of several types of cancer among Chinese people.

INTRODUCTION

It has been shown that the incidence of certain types of cancer varies in different parts of the world. For example, in Japan there is a high rate of stomach cancer, whereas in China nasopharyngeal and liver cancers are particularly common. When Japanese or Chinese people emigrate to the United States their offspring like other Americans show a higher incidence of colon cancer and breast cancer. In an attempt to find the aetiological factor(s) at the root of the most common cancers in China, the Ames method for detecting mutagens using *Salmonella typhimurium* (Ames, McCann & Yamasaki, 1975) was used to screen extracts of the foods particularly favoured by Chinese people. In the first stage of these investigations, we found that ether extracts of soya bean sauce treated with sodium nitrite at pH 3 were mutagenic for *S. typhimurium* strains TA1535 and TA100, and that ascorbic acid prevented the formation of the mutagenic substance (Lin, Wang & Yeh, 1979). In the present investigation, we report that some popular Chinese wine also contains substances that are mutagenic to *S. typhimurium* strains TA1535 and TA100 after incubation of the wine with nitrite.

EXPERIMENTAL

Materials. Glass fibre sheets impregnated with silica gel for thin-layer chromatography were purchased from Gelman Instrument Company (Ann Arbor, MI, USA). Glucose-6-phosphate and nicotinamide adenine dinucleotide were obtained from Calbiochem (San Diego, CA, USA). All chemicals used in these experiments were chemically pure or of analytical grade. The wine samples were purchased from the local stores in Taipei city.

Mutagenicity assay. The mutagenicity assay was performed according to the method of Ames *et al.* (1975). Ether extracts of samples were prepared by concentrating 600 ml of wine with a flush evaporator under reducing pressure to 200 ml. An aliquot of the condensate was adjusted to pH 3 and treated with 2000 ppm $NaNO_2$; the reaction was carried out at 25°C for 1 hr. It was terminated by adding ammonium sulphamate. The reaction product was then extracted twice with 200 ml of ether. The combined ether extracts were reduced under a stream of nitrogen to 0·5 ml and then diluted with 7·5 ml dimethylsulphoxide (DMSO) for the assay. In the control sample no sodium nitrite was added to the condensate. To find the effects of using various levels of nitrite, 1000, 2000, 5000, 10,000 or 15,000 ppm $NaNO_2$ was added to the condensate. When the reaction was complete, the reaction mixture was extracted with ether, and finally dissolved in 7·5 ml DMSO as described above. In this case a 0·1-ml sample of each DMSO extract was diluted ten times with DMSO for the assay. To determine the effects of ascorbic acid, 13,000 ppm ascorbic acid was added to the reaction mixture containing 5000 ppm sodium nitrite at pH 3.

Partial purification of mutagen by thin-layer chromatography (TLC). Ether extracts of wine A (equivalent to about 120 ml) pretreated with 2000 ppm $NaNO_2$ were chromatographed on a glass filter sheet impregnated with silica gel (Gelman ITLC-SG) using hexane–ether–dichloromethane (40:30:20, by vol.) as the chromatographic solvent system. The chromatograms were cut into 1-cm strips which were individually extracted with chloroform–methanol (90:10, v/v) and the extracts were dried under a stream of nitrogen gas. The residues were dissolved in DMSO and aliquots were tested using *S. typhimurium* strain TA100.

Free amino acid analysis. Aliquots of the wine samples were diluted with 0·2 M-sodium citrate, pH 2·2 and a Beckman (Palo Alto, CA, USA) model 120C amino acid analyser was used for the quantitative measurement of the free amino acid content of each sample according to the method of Spackman & Moor (1958).

RESULTS

The dose-response curve obtained from the ether extract of wine A treated with nitrite is shown in Fig. 1. Clearly, this extract contains mutagenic substances that are active towards *S. typhimurium* strain TA100. We also found that the activity of these substances determined using TA1535, TA1538 and TA98 was about one third to one sixth of that using TA100 and

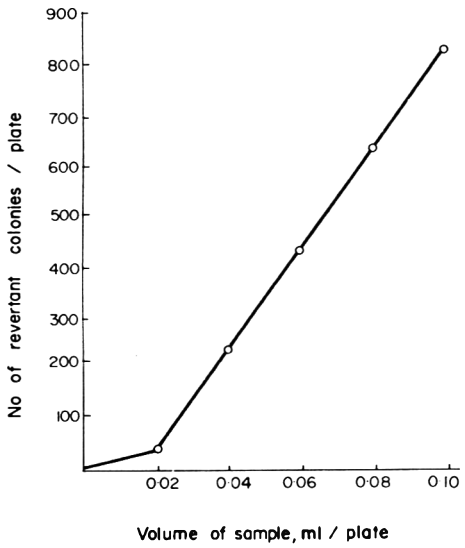

Fig. 1. Dose-response curve of ether extract of nitrite-treated wine A tested on strain TA100 of *S. typhimurium* according to the method of Ames *et al.* (1975).

that the mutagens were equally active in the presence or absence of a microsomal preparation. The number of revertants produced by the control sample not treated with nitrite was not significant. The formation of mutagens by nitrite treatment is pH-dependent; the optimal pH was 2·0 for *S. typhimurium* strains TA1535 and TA100. The effects of various nitrite levels on mutagen formation are shown in Fig. 2. In the presence of ascorbic acid, the formation of mutagenic materials was prevented.

The result of the partial purification of extracts of wine A by TLC are shown in Fig. 3. The nitrite-treated wine A gave a mutagenic active peak at R_F 0·72, using the hexane–ether–dichloromethane solvent system (Stahl & Schorn, 1969).

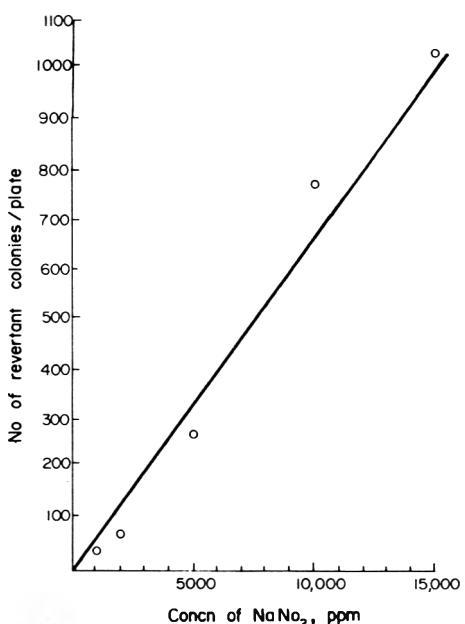

Fig. 2. Effect of nitrite concentration on the production in nitrite-treated wine B of material mutagenic to *S. typhimurium* TA100.

Table 1. *Amino acid composition of several kinds of Chinese wine**

Amino acid	Level of amino acid (mg/100 ml) in		
	Wine A	Wine B	Wine C
Lys	26·28	59·53	28·02
His	11·32	18·91	0
Arg	38·87	93·96	0
Asp	36·19	20·81	17·62
Thr	11·42	8·57	2·68
Ser	22·05	16·70	6·23
Glu	22·74	19·92	7·63
Pro	40·03	25·36	12·58
Gly	29·56	18·90	16·31
Ala	68·26	45·70	31·20
Cys(half)	0	0	0
Val	27·23	16·32	14·78
Met	7·35	3·05	0
Ile	17·29	11·59	8·69
Leu	41·05	13·49	5·32
Tyr	34·28	18·64	7·35
Phe	27·95	16·01	12·06

*None of the amino acids listed above were detected in the two distilled wines tested.

The results of the quantitative analysis for free amino acids in wine are summarized in Table 1. The amounts of free amino acids detected in the two distilled wines tested were not significant. Significant amounts of free amino acids were found in the non-distilled wines (A, B & C) tested.

DISCUSSION

The results indicate that the mutagen extracted from nitrite-treated wine A is of medium polarity as judged by its R_F value in the solvent (Stahl & Schorn, 1969), that the mutagen is directly mutagenic requir-

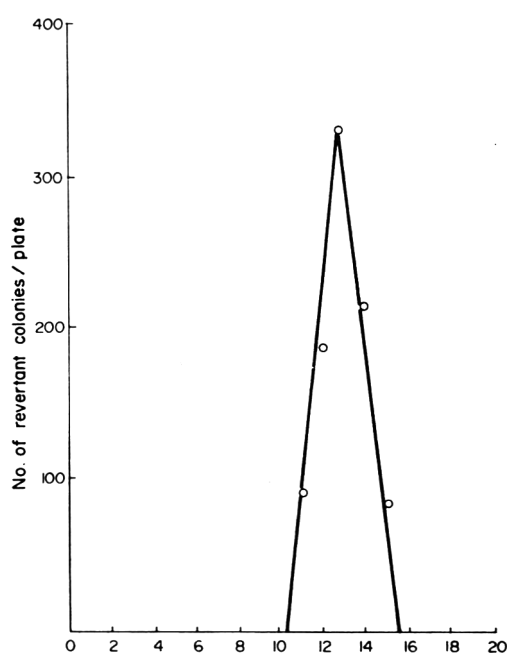

Fig. 3. Thin-layer chromatographic analysis of the ether extracts of nitrite-treated wine A.

ing no microsomal activation, and that the mutagen is formed rapidly by nitrosation at pH 2·0, suggesting that it is a nitrosamide (Mirvish, 1975).

The carcinogenicity of nitrosamines and nitrosamides was first established by Barnes & Magee (1954); their possible relevance to cancer in man was demonstrated when tumours were produced in animals by feeding amine and nitrite simultaneously (Druckrey, 1975; Lijinsky & Taylor, 1977; Sugimura & Kawasachi, 1973). It has been suggested that the nitrosatable products in fish may be an aetiological factor in stomach cancer in Japan (Neurath, Dünger, Pein, Ambrosius & Schreiber, 1977). The mutagenic strength of nitrite-treated raw fish and wine A was compared using *S. typhimurium* strain TA100 and it was found that 4·3 ml of wine A was equivalent to 1·0 g of raw fish, using the data for fish presented by Marquadt, Rubino & Weisburger (1977).

The use of nitrite in food processing is widespread in Taiwan (Chen & Lin, 1977; Lin, 1978) and Chinese wine is frequently served together with nitrite-treated sausage; this could contribute to the higher incidence of several types of cancer in Chinese people.

Of the 12 different types of Chinese wine tested, seven of the non-distilled wines were found to contain similar levels of mutagenic substances after treatment with nitrite. No mutagens were detected in wines made by distillation of fermented products when these wines were treated with nitrite. In order to study the differences between the two types of wine, the free amino acid content of some of the wines was measured. Significant levels of free amino acids were not detected in distilled wines but levels of 3–94 mg/100 ml of various free amino acids were found in the non-distilled wines. This strongly suggests that the mutagen precursor is a polar compound which is either present in the raw materials used to make wine or produced during fermentation.

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STUDIES OF THE EFFECT OF 2-(2′-HYDROXY-5′-METHYLPHENYL)BENZOTRIAZOLE ON RAT LIVER

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Abstract—The repeated oral administration of 2-(2′-hydroxy-5′-methylphenyl)benzotriazole, an ultraviolet-light absorber, in a dose of 300 mg/kg daily to young adult male rats for 14 or 28 days caused a reversible increase in relative liver weight. Microsomal glucuronosyltransferase activity was enhanced, whereas the activities of other drug-metabolizing enzymes, of glucose-6-phosphatase and of some lysosomal acid hydrolases were only marginally stimulated or remained essentially unchanged. Electronmicroscopic examination did not reveal any conspicuous proliferative response of the smooth endoplasmic reticulum in hepatocytes from the treated animals. After administration of a single oral 10-mg/kg dose of the test compound labelled with carbon-14, the radioactivity was almost completely eliminated from the body within 168 hr.

INTRODUCTION

2-(2′-Hydroxy-5′-methylphenyl)benzotriazole (UVA), an ultraviolet-light absorber, is incorporated into various plastics. This report presents the results of morphological and biochemical studies of the effects of repeated oral treatment with UVA on the rat liver.

EXPERIMENTAL

Test chemicals. 2-(2′-Hydroxy-5′-methylphenyl)-benzotriazole (UVA) was a light-yellow crystalline powder, m.p. 128–132°C. [^{14}C]UVA, labelled in the benzene ring and in the 5′-methyl group, was obtained from Natec (Hamburg, Federal Republic of Germany) and had a specific activity of 29·1 μCi/mg. For the present study, this preparation was diluted with unlabelled material to a specific activity of 5·07 μCi/mg. The radiochemical purity of the material was checked by inverse isotope-dilution analysis and found to be greater than 99%. Ethoxycoumarin was prepared as described by Ullrich & Weber (1972). Ethyl isocyanide was kindly supplied by Dr. J. Rody, Ciba-Geigy Ltd., Basel. All other reagents were of the highest purity available.

Animals. Male albino rats (RAI; mean body weight 80 g) from our specified-pathogen-free colony were kept in rooms maintained at 22 ± 1°C with a relative humidity of 55 ± 5% and a 12-hr light/dark cycle and were given a standard laboratory diet (Nafag 890, Gossau) and drinking-water *ad lib.*

Treatment. UVA, dissolved in corn oil, was administered to groups of ten animals by gavage in a daily dose of 300 mg/kg for 14 or 28 days. Comparable control groups received an equal volume of corn oil. The animals were killed 1 day after administration of the last dose. Recovery experiments were also performed in which the animals received 300 mg/kg for 14 days and were killed 28 days after withdrawal of UVA. Additional experiments were carried out to determine the *O*-dealkylation of ethoxycoumarin and the activities of NADPH-cytochrome *c* reductase and acid hydrolases; in these experiments groups of only four animals were used and no recovery experiments were carried out.

Preparation of subcellular fractions. At the termination of the treatment, the animals were weighed and bled, and their livers were removed, weighed and chilled in ice. The tissue was then homogenized for 1 min in 5 vols 1·15% KCl at 4°C in a glass homogenizer equipped with a motor-driven (1000 rpm) teflon pestle. The resultant homogenate (25%) was centrifuged for 20 min at 12,000 *g* (Sorvall RC-2) to yield a (postmitochondrial) supernatant and a pellet (MLP fraction). The postmitochondrial supernatant was spun for 1 hr at 105,000 *g* (Spinco Model L ultracentrifuge, 60 Ti rotor). The resultant pellet represented the microsomal fraction. For determination of the *O*-dealkylation of ethoxycoumarin the microsomal pellet was washed once with 1·15% KCl.

Biochemical determinations. The content of protein (Lowry, Rosebrough, Farr & Randall, 1951) and the activity of UDPglucuronosyltransferase (Mulder & van Doorn, 1975) and of biphenyl 4-hydroxylase (Creaven, Parke & Williams, 1965) were measured in the postmitochondrial supernatant. In the microsomal fraction, the following parameters were evaluated: the content of protein, phospholipid (Morrison, 1964) and cytochrome *P*-450 (Omura & Sato, 1964), and the activity of aminopyrine *N*-demethylase (Gram, Wilson & Fouts, 1968), ethylmorphine *N*-demethylase (Holtzman, Gram, Gigon & Gillette, 1968), 3-methyl-4-methylaminoazobenzene (3-MMAB) *N*-demethylase (Sladek & Mannering, 1969a) and glucose-6-phosphatase (Ricketts, 1963). The difference spectra of dithionite-reduced microsomes with ethyl isocyanide as a ligand for cytochrome *P*-450 were recorded essentially according to Imai & Siekevitz (1971), the ratio for the Soret band peaks at 455 and

430 nm being estimated at pH 7·4. 7-Ethoxycoumarin *O*-deethylase activity was determined essentially as prescribed by Ullrich & Weber (1972), in the presence or absence of the inhibitor, 7,8-benzoflavone (Ullrich, Frommer & Weber, 1973). Activities of β-glycerophosphatase (Gianetto & de Duve, 1955), *p*-nitrophenylphosphatase (Shibko & Tappel, 1963) and β-glucuronidase (Gianetto & de Duve, 1955) were assayed in MLP fractions.

Electron microscopy. Small tissue blocks were fixed for 2 hr in 1% phosphate-buffered (pH 7·4) osmium tetroxide (4°C) and embedded in Araldite. The ultrathin sections were double-stained with uranyl acetate and lead citrate and examined in a Philips EM 300 microscope.

Distribution and elimination studies. ^{14}C-Labelled UVA (5·07 μCi/mg) was administered to a group of four male albino rats (weighing about 200 g) in a single oral dose of 10 mg/kg dissolved in polyethylene glycol 400. Prior to treatment the animals were fasted overnight. During the experiment they were housed in metabolism cages and had free access to food and water. Urine and faeces were collected in 24-hr periods until 168 hr after dosage, when the animals were killed and organs and tissues were obtained by dissection. Radioactivity was determined with a TRI-CARB liquid scintillation spectrometer (Packard Model 3375), using the external-channel-ratio method to correct sample-quenching. Blood and tissue samples were analysed directly after solubilization with Soluene 100 (Packard Instrument Co., Frankfurt/Main, Federal Republic of Germany) and the addition of IRGASCINT (Ciba-Geigy AG). Prior to counting, faeces were combusted by the oxygen-flask technique described by Kalberer & Rutschmann (1961).

RESULTS

Body weight and relative liver weight

The repeated administration of UVA to young adult male rats had no influence on their growth rate, but caused an increase in the relative liver weight, which still persisted to some extent during the 28-day period after the discontinuation of treatment (Table 1).

Microsomal constituents

The microsomal protein content was slightly decreased after treatment with UVA for 14 days, but was significantly increased after the 28-day administration. However, it should be realized that, for unknown reasons, the control value in the latter case was considerably lower than that to be expected from the other control figures (Table 2). Microsomal phospholipid was unaltered after treatment with UVA (Table 2).

The activity of several mixed-function oxidases remained unchanged after the administration of UVA, as shown in Table 2. The content of cytochrome *P*-450 was similarly unaffected (Table 2). The carbon monoxide difference spectra of dithionite-reduced microsomes showed a maximum at 450 nm for treated as well as for control animals (data not shown). By using the ligand ethyl isocyanide, it is possible to distinguish spectrometrically between the 'phenobarbital-type' cytochrome *P*-450 and the 'polycyclic hydrocarbon-type' cytochrome *P*-448 (Imai & Siekevitz, 1971). Comparison with the spectra obtained from the controls revealed no effect of UVA on the Soret-peak 455/430 nm ratio of the ethyl isocyanide difference spectra of reduced liver microsomes. At pH 7·4, this ratio varied between 0·5 and 0·75 and was independent of the duration of treatment or the recovery period. Ullrich *et al.* (1973) showed that 7,8-benzoflavone specifically inhibited the *O*-dealkylation in rats pretreated with polycyclic hydrocarbons. It can be seen from Fig. 1 that 7,8-benzoflavone did not inhibit the activity of liver-microsomal ethoxycoumarin *O*-deethylase of control or phenobarbital- or UVA-treated animals. In contrast, the activity of this enzyme was markedly inhibited if 7,8-benzoflavone was added to liver microsomes of 3-methylcholanthrene-treated rats. Administration of UVA increased UDPglucuronosyltransferase activity, whereas that of glucose-6-phosphatase was slightly reduced, although only in the animals treated for 14 days (Table 2).

Acid hydrolases

As shown in Table 3, treatment with UVA had no appreciable effect on the free and total activity of the various acid hydrolases in MLP fractions.

Table 1. *Effects of 14 or 28 daily oral doses of 300 mg UVA/kg on body weight and relative liver weight of male rats*

Duration of treatment (days)	Treatment	Body weight (g)	Liver weight (% of body weight)
14	Control†	180 ± 13	4·61 ± 0·34
	UVA	172 ± 12	6·40 ± 0·28***
28	Control†	276 ± 17	4·32 ± 0·23
	UVA	276 ± 15	5·28 ± 0·34***
14 (+28‡)	Control†	357 ± 26	3·76 ± 0·23
	UVA	384 ± 32	4·46 ± 0·25***

UVA = 2-(2′-Hydroxy-5′-methylphenyl)benzotriazole

†Corn oil treatment.

‡After treatment, animals were kept for 28 days without treatment before being killed.

Values are means ± SD for groups of ten rats and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control value: ***$P < 0·001$.

Table 2. *Effects of 14 or 28 daily oral doses of 300 mg UVA/kg on various hepatic microsomal parameters in the male rat*

Parameter	Mean values following treatment for					
	14 days		28 days		14 days + 28 days recovery	
	Control	UVA	Control	UVA	Control	UVA
Microsomal protein (mg/g liver)	15·89 ± 1·41	13·96 ± 2·71	11·64 ± 1·10	17·87 ± 1·22***	14·96 ± 1·02	14·22 ± 1·61
Microsomal phospholipid (mg/g liver)	5·22 ± 0·62	5·08 ± 0·68	4·54 ± 0·62	4·70 ± 0·54	5·41 ± 0·31	5·09 ± 0·54
Cytochrome *P*-450 (nmol/g liver)	3·15 ± 0·48	3·42 ± 0·59	3·30 ± 0·40	3·54 ± 0·82	3·68 ± 1·10	3·74 ± 0·99
Ethylmorphine *N*-demethylase (μmol HCHO formed/hr/g liver)	5·94 ± 1·44	5·91 ± 1·30	6·48 ± 1·36	6·50 ± 1·30	11·80 ± 2·46	12·13 ± 2·23
Aminopyrine *N*-demethylase (μmol HCHO formed/hr/g liver)	0·92 ± 0·28	0·83 ± 0·31	0·91 ± 0·25	1·18 ± 0·17*	1·23 ± 0·25	1·35 ± 0·22
3-MMAB *N*-demethylase (μmol HCHO formed/hr/g liver)	5·15 ± 1·53	3·54 ± 1·70	3·89 ± 1·30	4·21 ± 2·01	7·78 ± 1·10	7·17 ± 1·27
Biphenyl 4-hydroxylase (μmol 4-hydroxybiphenyl formed/hr/g liver)	0·606 ± 0·063	0·590 ± 0·083	0·630 ± 0·064	0·600 ± 0·066	0·631 ± 0·034	0·635 ± 0·065
UDPglucuronosyltransferase (μmol UDP formed/hr/g liver)	10·10 ± 2·71	28·00 ± 10·77**	10·13 ± 2·12	19·49 ± 5·12***	8·59 ± 2·32	10·24 ± 4·07
Glucose 6-phosphatase (mmol P formed/hr/g liver)	1·52 ± 0·17	1·26 ± 0·15**	1·47 ± 0·12	1·47 ± 0·08	1·27 ± 0·03	1·50 ± 0·22

UVA = 2-(2′-Hydroxy-5′-methylphenyl)benzotriazole 3-MMAB = 3-Methyl-4-methylaminoazobenzene

Values are mean ± SD for groups of ten rats and those marked with asterisks differ significantly (by Student's *t* test) from the corresponding control value: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

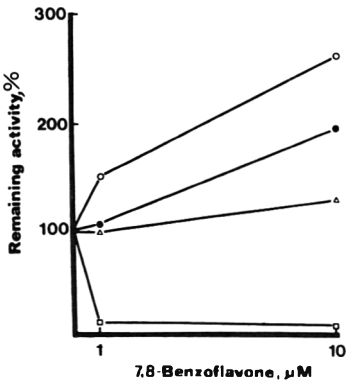

Fig. 1. Ethoxycoumarin *O*-deethylase activity measured in microsomal fractions at 30°C in the presence of 0·1 mM-ethoxycoumarin, 0·15 mM-NADPH, 0·1 M-Tris-HCl buffer (pH 7·4), suitable amounts of microsomal protein (about 0·1 mg/ml) and different concentrations of 7,8-benzoflavone. The microsomal fractions were pooled from groups of four animals treated with corn oil (○; control), 14 doses of 300 mg UVA/kg (●), three doses of 80 mg sodium phenobarbital/kg (△) or two doses of 20 mg 3-methylcholanthrene/kg (□). The specific activities (in nmol/min/mg protein) in the absence of 7,8-benzoflavone were: control 0·7, UVA 1·0, sodium phenobarbital 4·0, and 3-methylcholanthrene 9·5.

Electron microscopy

The subcellular organization of hepatocytes from control animals given corn oil for 14 days is shown in Fig. 2a. Comparison of Figs 2a and 2b demonstrates that treatment with UVA for 14 or 28 days did not produce any major alterations in the organelle pattern of the hepatocytes, and irrespective of the duration of treatment, the proliferative response of the smooth endoplasmic reticulum was moderate compared with that observed in the hepatocytes from animals repeatedly treated with phenobarbital as a liver-inducing agent (Remmer & Merker, 1965; Stäubli, Hess & Weibel, 1969). In animals killed 28 days after the discontinuation of treatment with UVA, the liver parenchymal cells exhibited a normal organelle pattern (results not presented).

UVA distribution and elimination studies

Within 48 hr of administration of a single oral dose of 10 mg [^{14}C]UVA/kg, about 91% of the radioactivity administered was eliminated from the body. Within 168 hr, the radioactivity had been almost completely recovered, about 73% having been found in the urine and 27% in the faeces. By that time, the residual radioactivity measured in the tissues was below the blood level of 0·017 μg/g, except in the kidney, the aorta and the liver (0·10–0·22 μg/g). The highest level of activity was present in the liver, which contained 0·1% of the initial dose.

DISCUSSION

Although daily oral treatment of male rats with 300 mg UVA/kg for 14 consecutive days caused an increase in the relative liver weight (Table 1), no drastic change in the activity of various drug-metabolizing enzymes occurred; the activity of UDPglucuronosyltransferase, however, was enhanced (Table 2). The rise in some microsomal parameters, notably aminopyrine *N*-demethylase activity, when treatment was prolonged to 28 days, was observed whether the activities were expressed per gram of liver tissue (as in Table 2) or per milligram of microsomal phospholipid. When microsomal protein was chosen as a basis, most of the specific activities of UVA-treated rats were below those of controls, but this finding was probably due to the unexpectedly low recovery of microsomal protein in the 28-day controls. The endoplasmic reticulum was only affected to a slight extent, as was confirmed by the absence of any extensive proliferation of smooth membranes (Figs 2a,b). After treatment had been discontinued for 28 days, the values for the microsomal parameters were similar to those of the controls although the relative liver weight was still somewhat elevated.

Certain 2-*tert*-butylphenol derivatives were reported by Golberg (1966) to induce a pattern of changes characterized by enlargement of the liver and an unchanged or decreased activity of monooxygenases and glucose-6-phosphatase. This pattern was somewhat similar to that induced by UVA. Golberg (1966) argued that drug-induced hepatomegaly not associated with increased microsomal monooxygenase activity might be indicative of toxic liver damage. Typical hepatotoxins such as coumarin (Feuer, Cooper, de la Iglesia & Lumb, 1972; Feuer, de la Iglesia & Cooper, 1974; Lake, Longland, Hodgson, Severn & Gangolli, 1977), thioacetamide (Barker & Smuckler, 1972; Feuer, Golberg & Le Pelley, 1965; Feuer *et al.* 1972 & 1974; Platt & Cockrill, 1969) and

Table 3. *Effects of 14 daily oral doses of 300 mg UVA/kg on the activity of various acid hydrolases in MLP fractions*

Parameter		Mean enzyme activities in		Activity in test group as % of control
		Controls	UVA group	
Protein (mg/g liver)		32·02 ± 2·12	33·80 ± 2·56	106
β-Glycerophosphatase (P*):	Free	0·089 ± 0·016	0·078 ± 0·009	88
	Total	0·236 ± 0·017	0·227 ± 0·014	96
p-Nitrophenolphosphatase (nitrophenol*):	Free	0·077 ± 0·003	0·081 ± 0·009	105
	Total	0·091 ± 0·005	0·086 ± 0·004	95
β-Glucuronidase (phenolphthalein*):	Free	0·036 ± 0·003	0·031 ± 0·004	86
	Total	0·081 ± 0·006	0·071 ± 0·005	88

*In mg of identified reaction product formed/10 min/g liver.
Values are means ± SD for groups of four animals.

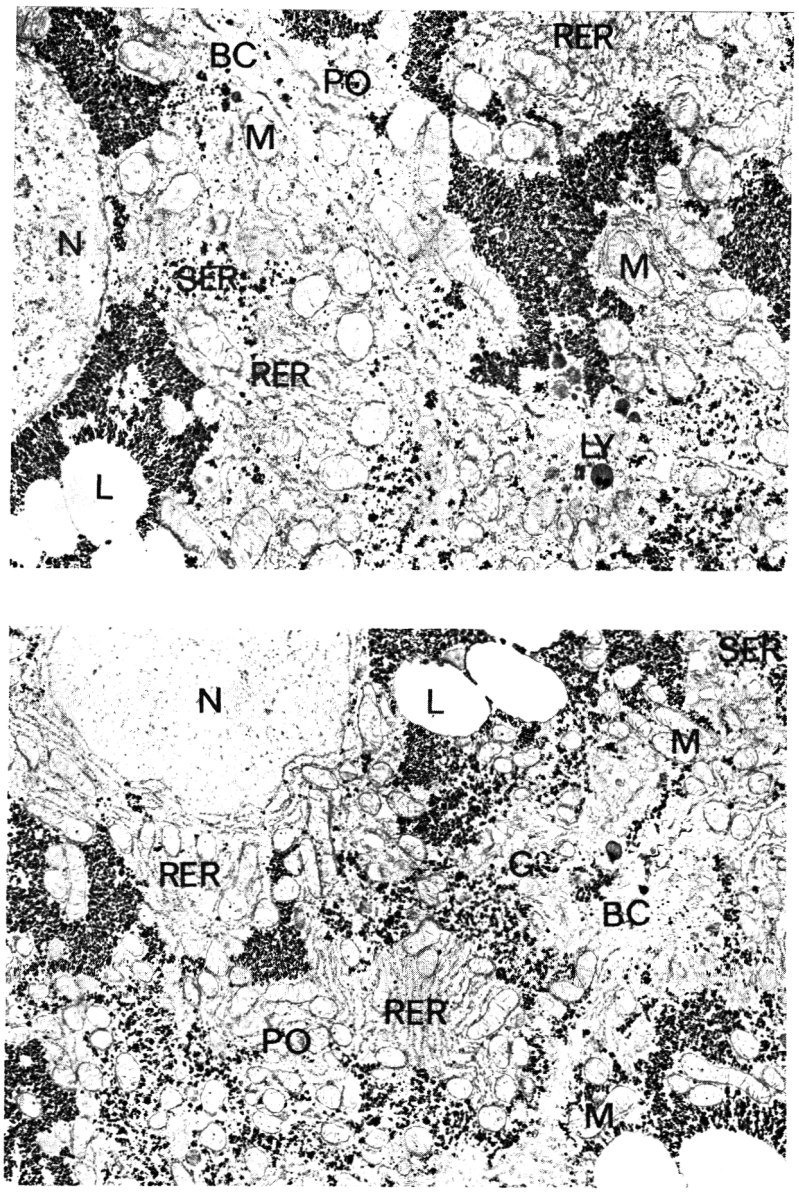

Fig. 2. (a) Usual organelle pattern of rat hepatocytes in micrograph from control rat treated with corn oil for 14 days, and (b) similar appearance of hepatocyte from a rat given 300 mg UVA/kg/day for 14 days, showing the endoplasmic reticulum formed by rough (RER) and smooth (SER) membranes, the latter associated with glycogen particles, Golgi membranes organized in stacks (G), cytoplasmic particles identified as mitochondria (M), peroxisomes (PO) and peribiliar dense bodies (LY), part of the nucleus (N), bile canaliculi (BC) and lipid vacuoles (L). × 14,625.

acetaminophene (Thorgeirsson, Sasame, Mitchell, Jollow & Potter, 1976) can provoke a significant reduction in the activity or content of several constituents of the microsomal drug-metabolizing system, but these signs of liver injury were not produced by UVA. Furthermore, there was no evidence of any intrahepatic accumulation of UVA or its metabolites. The results of the elimination and distribution studies indicated that, upon oral administration, UVA was rapidly and almost completely eliminated from the organism. It has also been suggested that a reduction in the activity of glucose-6-phosphatase (Golberg, 1966) and an increase in the activity of lysosomal hydrolases (Grasso, 1976) are both pathognostic manifestations of hepatotoxicity. The level of glucose-6-phosphatase was slightly reduced after the administration of UVA for 14 days and showed no further change when the treatment was prolonged to 28 days (Table 2). The free and total activities of some selected acid hydrolases were not altered (Table 3). In addition, electronmicroscopic inspection of the liver tissue did not reveal any major changes in the structure of the hepatocyte constituents.

Various microsomal parameters were investigated to compare the enzyme-induction pattern elicited by UVA with that known to occur in rats after the administration of carcinogenic polycyclic hydrocarbons. Some of these agents cause *de novo* formation of a particular subspecies of cytochrome *P*-450, referred to as cytochrome *P*-448 or P_1-450 (Mannering, 1971). Cytochrome *P*-448 can be distinguished from cytochrome *P*-450, which is characteristically present in the microsomes of untreated or phenobarbital-treated animals, by the difference spectra of dithionite-reduced microsomes obtained either with carbon monoxide (Mannering, 1971) or with ethyl isocyanide (Imai & Siekevitz, 1971) as a ligand for the haem protein. Our experiments with carbon monoxide and ethyl isocyanide showed that treatment of rats with UVA did not induce detectable amounts of cytochrome *P*-448. The carbon monoxide difference spectra showed the usual maximum at 450 nm. Furthermore, the ethyl isocyanide spectra of treated animals did not differ from those of the control animals; the Soret-peak (455/430 nm) ratios were smaller than 1 (0·5–0·75), whereas in rats treated with 3-methylcholanthrene the values normally lie between 1·2 and 1·5 at pH 7·4 (Alvares, Bickers & Kappas, 1973; Imai & Siekevitz, 1971; Sladek & Mannering, 1969b). 7,8-Benzoflavone proved to be a rather specific inhibitor of ethoxycoumarin *O*-deethylation if assayed with microsomes of rats treated with either benzo[*a*]pyrene or 3-methylcholanthrene (Ullrich *et al.* 1973). Our results confirmed those findings and, furthermore, indicated that the addition of 7,8-benzoflavone to liver microsomal fractions prepared from control or phenobarbital- or UVA-treated rats stimulated the *O*-deethylation of ethoxycoumarin (Fig. 1). Phenobarbital and polycyclic hydrocarbons were reported to increase the activity of 3-MMAB *N*-demethylase (Sladek & Mannering, 1969a), but UVA did not have any such effect. From these results it is concluded that UVA did not produce an enzyme-induction pattern in hepatocytes comparable to that produced by polycyclic aromatic hydrocarbons. These findings concur with the results obtained after long-term feeding of UVA to rats; no evidence of any tumorigenic activity was recorded (B. Hunter, C. Graham, A. E. Street, R. Heywood and C. P. Cherry, unpublished report submitted to Ciba-Geigy AG, Basel, 1975).

Treatment with UVA induced a response pattern characterized by an apparent dissociation between liver enlargement and the stimulation of the microsomal mono-oxygenase system. It should be recalled that the members of the vast family of liver-inducing xenobiotics display great differences in the extent to which they provoke hepatomegaly and induce drug-metabolizing or other enzymes (Schulte-Hermann, 1974). In keeping with this observation, we suggest that UVA is a liver-inducing agent that can exert a slight stimulant effect on the formation of mixed-function oxidases. This interpretation is supported by the finding that the activities of two microsomal enzymes, UDPglucuronosyltransferase and aminopyrine *N*-demethylase, are stimulated by prolonged (28-day) treatment with UVA.

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METABOLISM AND TISSUE DISTRIBUTION OF [^{14}C]AFLATOXIN B_1 IN PIGS

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Abstract—Excretion of radioactive aflatoxin B_1 and its metabolites over a 9-day period following the oral administration of ring-labelled [^{14}C]aflatoxin B_1 was investigated in two pigs. The major excretory route was found to be the faeces (accounting for 51 and 65% of the dose in the two pigs). Less than 20% of the dose was excreted in the urine. Only about 4% of the dose was recovered in the faeces and urine in identifiable forms, mainly aflatoxin M_1 and unchanged aflatoxin B_1 with some traces of aflatoxicol. In the blood plasma most of the radioactivity was bound to proteins, the biological half-life of these products being approximately 6 days. Tissue analyses demonstrated that the highest ^{14}C activities were in the liver, kidney and lung 1 and 2 days after dosage. More than 90% of the radioactivity in the liver tissue was neither dialysable nor extractable with methylene chloride.

INTRODUCTION

Aflatoxins are potent liver carcinogens produced by some strains of the ubiquitous moulds *Aspergillus flavus* and *A. parasiticus*. They have been reported as contaminants in a number of human and animal foodstuffs (Armbrecht, 1972) and have been found most frequently in ground-nut cake, often used as a protein source for livestock. Indirectly, the presence of aflatoxins in such materials poses a problem for human health because of the possible carry-over of dietary aflatoxins or their metabolites into foodstuffs of animal origin, such as milk, meat and eggs (Purchase, 1972).

To understand these connections it is of great importance to elucidate the metabolic fate and kinetics of aflatoxins in farm animals. Although the susceptibility of different animal species to aflatoxin B_1 (AFB_1) is very variable, the common feature is that AFB_1 is generally easily absorbed and extensively metabolized, only a few percent or less of the applied dose having been found to be excreted unchanged in all the animals tested so far. The possible transformations of AFB_1 in animals—as far as they are known—are extensively discussed in a review by Campbell & Hayes (1976).

For practical reasons it is convenient to classify AFB_1-related compounds into three different groups:

(1) Methylene chloride- or chloroform-soluble compounds (aflatoxins B_1, M_1, R_0, P_1, Q_1, B_{2a}, H_1 and MQ_1);
(2) Low molecular, water-soluble compounds (glucuronides and sulphates of aflatoxins M_1, R_0, P_1, Q_1, H_1 and MQ_1, and the glutathione-adduct of AFB_1 2,3-oxide);
(3) Metabolites covalently bound to cellular macromolecules like proteins, RNA and DNA (derivatives of AFB_1 2,3-oxide, AFB_{2a} and AFB_1 2,3-dihydrodiol).

The most dangerous compounds are likely to be found in group 1, but insufficient toxicological data on compounds in groups 2 and 3 are at present available.

To evaluate fully the carcinogenic risk of these compounds for man it would be necessary also to consider data on the carcinogenic potency of the various metabolites and their concentration in different edible tissues. Published reports on the fate of AFB_1 in pigs are scarce and the studies are limited to the detection of residues of unchanged AFB_1 and of AFM_1 in different tissues after the feeding of diets containing 0·1–1·0 ppm AFB_1 (Jacobson, Harmeyer, Jackson, Armbrecht & Wiseman, 1978; Jemmali & Murthy, 1976; Keyl & Booth, 1971; Krogh, Hald, Hasselager, Madson, Martensen, Larsen & Campbell, 1973). Highest concentrations were found in the liver and kidneys, with lower levels in the muscles and blood. Generally the residues of AFB_1 and AFM_1 barely exceeded 1 μg AFB_1/kg wet weight of tissue. However, nothing is known about the occurrence and kinetics of other AFB_1-derived metabolites in pigs or about the withdrawal period needed for pigs on aflatoxin-contaminated diets. The experiments described below represent a contribution to the solution of these problems.

EXPERIMENTAL

Test material. [^{14}C]AFB_1 was prepared biosynthetically from sodium [1-^{14}C]acetate using *Aspergillus parasiticus* ATCC 1551, as outlined by Hsieh & Mateles (1971). The aflatoxin fraction contained 1–2% of the original radioactivity. The purity and identity of the product were checked spectrophotometrically and by repeated thin-layer chromatography in different solvent systems (see below) by comparison with authentic AFB_1. The specific activity of AFB_1 from different batches was in the range 15–63 μCi/μmol. with a radiochemical purity above 95%. AFB_1 and AFM_1 were purchased from Senn Chemicals, Dielsdorf. The two isomers of aflatoxicol (AFR_0) were prepared by reduction of AFB_1 with zinc borohydride according to the method of Hsia & Chu (1977). The

ultraviolet and mass spectra were in agreement with the published data.

Animals and treatment. Two female piglets (Hampshire × Deutsches Edelschwein) were obtained from the Kantonales Tierspital, Zürich. The animals (weighing 10–15 kg) were housed in metabolism cages (40 × 55 × 50 cm) allowing separate collection of faeces and urine. A polyethylene catheter was implanted in the carotid artery of one animal and taken out through the dorsal skin. To prevent blood clotting, the catheter was filled with heparin solution (5000 U/ml) and sealed by heating after blood removal. To study the excretion pattern the piglets received 5 μg (0·3 μCi) [^{14}C]AFB$_1$/kg body weight orally in capsules. Tissue distribution was studied 3 wk later in the same piglets each given a similarly encapsulated dose of 3·1 μg (0·2 μCi)/kg and killed 1 or 2 days later.

Excretion, metabolism and distribution studies. Urine and faeces were collected at 24-hr intervals throughout a 9-day period. Aliquots of the urine samples were counted directly in Insta-Gel (Packard Instruments, Downers Grove, IL, USA). Faeces were first added to a small portion of distilled water and mixed to ensure homogeneous distribution of radioactivity. Aliquots were incubated with tissue-solubilizer Soluene (Packard Instruments), mixed with Insta-Gel and counted in a Betaszint 5000 (Laboratorium Prof. Dr. Berthold, Wildbad, Federal Republic of Germany).

Samples of arterial blood were taken 3 and 7 hr and 1, 2, 6 and 10 days after treatment. Blood plasma was extracted twice with methylene chloride and an aliquot of the organic layer was counted. To distinguish between high- and low-molecular metabolites, the aqueous layer was dialysed and the radioactivity of each of the two fractions was determined separately.

Radio-thin-layer chromatography of methylene chloride extracts of urine and faeces was performed with the following developing solvents: (1) chloroform–acetone–water, 88:12:0·2 (by vol.); (2) ethyl acetate–chloroform, 75:25 (v/v); (3) chloroform–acetone–isoamyl alcohol, 80:10:10 (by vol.).

In the second (tissue-distribution) experiment, one piglet was killed 24 hr after dosing and the other at 48 hr. Organs and tissues were homogenized and 50–150-mg aliquots were treated with Soluene tissue solubilizer before counting.

RESULTS

Excretion pattern

The faeces constituted the major route of excretion (Table 1), accounting for 51·3 and 64·9% of the administered ^{14}C activity in the two pigs after 9 days. Only a small proportion (3·8 and 6·5% of the dose, respectively) was extractable with methylene chloride. Most of the radioactivity was excreted during the first 3 days, after which not more than 1–3% of the initial dose appeared daily.

The radioactivity found in the urine totalled 15·7 and 9·6% in the two pigs and most was excreted within the first 24 hr. The amount of methylene chloride-extractable activity accounted for 4·4 and 0·5% of the dose.

Table 1. *Radioactivity excreted in the faeces and urine of two piglets given a single oral dose of ^{14}C-labelled aflatoxin B_1*

Days after treatment	Percentage of dose excreted by			
	Pig 1		Pig 2	
	Total	Extractable*	Total	Extractable*
	Faeces			
1	13·6	2·3	24·3	3·8
2	18·4	1·3	19·8	1·4
3	8·0	0·2	9·1	0·2
4	3·7	0·01	3·3	1·1
5	1·0	ND	2·2	ND
6	1·8	ND	1·1	ND
7	2·4	ND	2·0	ND
8	1·3	ND	2·3	ND
9	1·1	ND	0·8	ND
0–9	51·3	3·8	64·9	6·5
	Urine			
1	12·3	3·6	6·6	0·4
2	1·3	0·7	1·0	0·1
3	0·7	0·07	0·5	0·02
4	0·3	0·06	0·4	0·01
5	0·3	ND	0·4	ND
6	0·3	ND	0·2	ND
7	0·2	ND	0·2	ND
8	0·2	ND	0·2	ND
9	0·1	ND	0·1	ND
0–9	15·7	4·4	9·6	0·5
	Faeces + urine			
	67·0	8·2	74·5	7·0

ND = Not detected (detection limit <0·005%)
*Extractable with methylene chloride.
Each piglet received 5 μg (0·3 μCi) [^{14}C]AFB$_1$/kg body weight by capsule.

Identification of metabolites

Radio-thin-layer chromatography of the methylene chloride-soluble compounds with solvent systems 1 and 3 indicated the presence of AFM$_1$ and AFB$_1$ in the urine and faeces. In solvent system 2, a small part of the radioactivity showed the same chromatographic behaviour as the less polar isomer of aflatoxicol. This metabolite was found in the faeces and to a lesser extent in the urine. AFM$_1$ accounted for about 50% of the methylene chloride-soluble radioactivity in the urine and for 15% in faeces, with AFB$_1$ accounting for 20 and 35%, respectively, and aflatoxicol for a total of less than 5% in each case. Unidentified products of greater polarity accounted for the rest of the activity.

Kinetics of AFB$_1$ metabolites in blood plasma

A marked level of radioactivity was already present in the serum of the blood sample taken 3 hr after the administration of [^{14}C]AFB$_1$ to the piglet with the carotid catheter (Fig. 1). Of this activity, 20% was extractable with methylene chloride. The low-molecular-weight water-soluble AFB$_1$ metabolites comprised only 10% of the activity, the dominant part (70%) being due to aflatoxins bound to macromole-

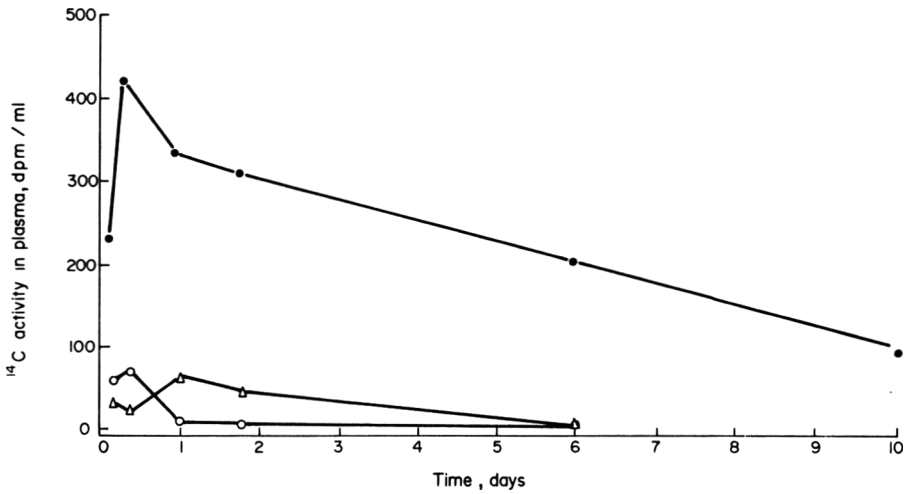

Fig. 1. Kinetics of methylene chloride-extractable (○), low-molecular-weight and water-soluble (△) and high-molecular-weight (●) [^{14}C]aflatoxin B_1 metabolites in the blood plasma of a pig after oral administration of 5 μg (0·3 μCi) [^{14}C]AFB_1/kg body weight.

cules. The highest level of radioactivity was attained during day 1. After 10 days, the plasma still contained 20% of the maximum level of radioactivity because of the very slow elimination of the high-molecular-weight AFB_1 metabolites.

Distribution of radioactivity in tissues and organs

The distribution of radioactivity in the tissues of two piglets 1 and 2 days after the second application of [^{14}C]AFB_1 is shown in Table 2. In each case the highest activity was found in the liver. Less than 0·2% of this labelled material was soluble in methylene chloride and less than 7% was water-soluble and dialysable. The tissue with the second highest level of radioactivity was the kidney, but the levels here were only 7–14% of those in the liver.

DISCUSSION

The excretion pattern of [^{14}C]AFB_1-derived radioactivity in piglets is similar to that found in other species, including the rat (Wogan, Edwards & Shank, 1967), mink (Chou & Marth, 1976) and rhesus monkey (Dalezios & Hsieh, 1973). Typically there is almost complete metabolism with excretion predominantly in the faeces, as a result of biliary secretion. The most important methylene chloride-soluble radioactive compounds excreted were AFM_1, the

Table 2. *Distribution of radioactivity in various tissues of piglets 1 and 2 days after oral administration of ^{14}C-labelled aflatoxin B_1*

	Residual radioactivity at			
	24 hr*		48 hr*	
Sample	dpm/g tissue	AFB_1 equivalents (ppb)	dpm/g tissue	AFB_1 equivalents (ppb)
Liver				
Total activity	2460	17·3	2450	17·3
H_2O-soluble and dialysable activity	160	1·1	100	0·7
CH_2Cl_2-soluble activity	<5	<0·04	<5	<0·04
Bile	190	1·3	418	2·9
Kidney	345	2·4	180	1·3
Lung	145	1·0	100	0·7
Heart	67	0·5	75	0·5
Spleen	53	0·4	60	0·4
Fat	74	0·5	27	0·2
Brain	<30	<0·2	<30	<0·2
Muscle	<30	<0·2	<30	<0·2

*Time after oral dosing of 15-kg piglets with 46 μg [^{14}C]AFB_1 (3·1 μg (0·2 μCi)/kg body weight).

combined urinary and faecal excretion of which accounted for 1·3 and 2·8% of the administered dose, and unchanged AFB_1 (2·2 and 2·4% in the two piglets). These amounts are comparable with the 4–9% AFM_1 and 1–4% AFB_1 excreted by the cow (Allcroft, Roberts & Lloyd, 1968), sheep (Nabney, Burbage, Allcroft & Lewis, 1967) and wether (Stoloff, Dantzman & Armbrecht, 1971). However, Dalezios, Hsieh & Wogan (1973) found a much greater AFM_1 excretion, approximately 20% of the dose, in the urine and faeces of rhesus monkeys.

The large amount and persistence of AFB_1 metabolites bound to macromolecules in the blood and liver are particularly notable. The structure of the metabolites has not been elucidated. Earlier studies conducted *in vitro* and *in vivo* with other animal species revealed that AFB_{2a} and AFB_1 epoxide may be the reactive intermediates capable of binding with various tissue components (Ashor & Chu, 1975; Essigmann, Croy, Nadzan, Busby, Reinhold, Büchi & Wogan, 1977; Patterson & Roberts, 1970; Swenson, Lin, Miller & Miller, 1977; Swenson, Miller & Miller, 1973). In the rat and monkey most of this retained radioactivity was found to be associated with the protein. The slow decrease of this plasma-bound radioactivity (half-life approximately 6 days) in the pig is similar to the corresponding data reported by Dalezios *et al.* (1973) for monkeys.

The distribution of AFB_1-derived radioactivity in the pig shows that the liver and kidney are the most susceptible edible tissues, whereas the residue levels in muscle meat are at least 80 times lower than those in the liver. Sawhney, Vadehra & Baker (1973) and Mabee & Chipley (1973) found a much more equal tissue distribution of [^{14}C]AFB_1 in chickens.

The further fractionation of aflatoxin residues in liver and blood revealed that the group 1 metabolites (see p. 253) are quantitatively not important, whereas compounds of group 2 and especially of group 3 are dominant. A possible method for estimating the carcinogenic potency of these latter compounds is presented in the next paper (Jaggi, Lutz, Lüthy, Zweifel & Schlatter, 1980).

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IN VIVO COVALENT BINDING OF AFLATOXIN METABOLITES ISOLATED FROM ANIMAL TISSUE TO RAT-LIVER DNA*

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Abstract—Ring-labelled [^{14}C]aflatoxin B_1 (AFB_1), prepared by biosynthesis, or generally labelled [^{3}H]AFB_1 was administered by oral gavage to young adult male rats. After 6 hr, the liver was removed and two fractions were isolated, namely macromolecules, which contained about 3% of the initial dose of AFB_1 radioactivity, and water-soluble, low-molecular aflatoxin conjugates containing about 0·2% of the administered radioactivity. These two fractions were administered orally to other rats in order to determine the potential of radioactive aflatoxin residues for covalent binding to DNA. Such binding can be used as an indicator for carcinogenic potency. Liver DNA was isolated 9–12 hr after administration of the aflatoxin derivatives and in no case was any radioactivity detected on the DNA. It can be deduced on the basis of the limit of detection of radioactivity on the DNA, that macromolecule-bound AFB_1 derivatives are at least 4000 times less active than AFB_1 with respect to covalent binding to rat-liver DNA, and that the water-soluble conjugates are at least 100 times less potent than AFB_1 itself. It is concluded that the carcinogenic risk for humans who consume liver or meat containing such aflatoxin residues is negligible when compared with the risk from intake of aflatoxins in other food items.

INTRODUCTION

Aflatoxins are among the strongest hepatocarcinogens. Their mode of carcinogenic action is probably based upon a covalent binding of metabolites to biological macromolecules, and there is increasing evidence that DNA is the most critical target.

Although interaction with DNA appears to be the most important factor in tumour initiation, binding to protein and RNA occurs more frequently. A few hours after administration of a single dose of aflatoxin B_1 (AFB_1) to a rat, less than 10% of the total macromolecule-bound metabolites is on the DNA, the rest being bound to RNA and to protein (Garner & Wright, 1975). The preceding paper (Lüthy, Zweifel & Schlatter, 1980) showed that the total macromolecule-bound radioactivity in the liver 24 hr after administration of a single dose of AFB_1 to a pig represented more than 10% of the dose. About 1% of the dose was accounted for by water-soluble, low-molecular-weight aflatoxin metabolites, probably conjugates with glucuronic acid, sulphate or glutathione, and only traces of lipophilic metabolites could be detected.

Since aflatoxins are regular contaminants of groundnut cake used in animal nutrition, aflatoxin residues (including macromolecule-bound aflatoxins and low-molecular conjugates) may be present in foods of animal origin (Purchase, 1972) and may be taken up by humans who consume the livers of these animals. It is possible that aflatoxin metabolites are cleaved from the macromolecules or from the conjugating agent during the process of digestion and are absorbed from the human intestine. No study is available on the carcinogenicity of such aflatoxin derivatives because a long-term carcinogenicity experiment would require considerable effort in the isolation of these derivatives from the livers of aflatoxin-treated animals and their subsequent feeding to other groups of animals.

In this report, the carcinogenicity of macromolecule-bound aflatoxin metabolites and of aflatoxin conjugates is estimated on the basis of a short-term system. DNA binding of carcinogens has been shown to be a useful quantitative measure for the carcinogenic potency of genotoxic compounds (Lutz, 1979), the binding potency being expressed by the so-called 'covalent binding index' (CBI), which shows how many molecules of a chemical are bound covalently per 10^6 DNA nucleotides after the administration of a single dose of 1 mmol/kg body weight. For rat-liver DNA, it was shown that the CBI is of the order of thousands with strong hepatocarcinogens, hundreds with moderate hepatocarcinogens and tens with weak carcinogens. This quantitative approach is especially suitable for a comparison of structurally related compounds, such as aflatoxins and their macromolecule-bound or conjugated metabolites.

We have therefore administered AFB_1 labelled with ^{14}C or ^{3}H to rats by gavage, isolated the macromolecules and the water-soluble conjugates of AFB_1 from the liver and administered these fractions orally to other rats for a determination of incorporation of radioactivity into the liver DNA of the second group of rats.

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EXPERIMENTAL

Materials and general methods. [^{14}C]AFB_1 was pre-

pared from sodium [1-^{14}C]acetate using *Aspergillus parasiticus* ATCC 1551, as outlined by Hsieh & Mateles (1971) and described by Lüthy *et al.* (1980). [^{3}H]AFB_1, obtained from Moravek Biochemicals, City of Industry, CA, USA, was specified by the distributor to have a specific activity of 20 Ci/mmol and a radiochemical purity of 98% as determined by thin-layer chromatography on silica gel G with chloroform–acetone, 9:1 (v/v). In aqueous media, however, we found that the tritium label was easily exchanged; 24 hr in an aqueous buffer at neutral pH resulted in a 15% loss of the label, and 37% was lost within 4 days. Because of this rapid exchange, our AFB_1 dose to the rat contained 50% of the tritium as tritiated water (HTO), so that it was necessary to perform the control experiment described below.

Animals and treatment. Young adult male rats [ZUR:SIV-Z] were used throughout. The aflatoxins were administered in 10% aqueous ethanol by stomach tube. The aflatoxin metabolites isolated from the livers of the sponsor rats were administered by stomach tube in the solvents indicated below. The animals were killed by open-heart puncture under ether anaesthesia.

Isolation of water-soluble aflatoxin metabolites from rat liver. Liver was homogenized in 3 vols water in a Waring Blender for four periods of 30 sec at high speed. After centrifuging for 1 hr at 12,000 g, the pellet was washed with 6 ml water and centrifuged again, the two supernatants being combined after filtration through paper. In order to separate the low-molecular conjugates from the macromolecule-bound metabolites the supernatant was dialysed three times at 4°C against 150 ml water.

The high-molecular content of the dialysis bag was extracted with methylene chloride and centrifuged for 30 min at 12,000 g (three times), and the aqueous phase, together with the glutinous layer, was lyophilized. The residue was taken up in 20 ml water and lyophilized again, and this procedure was repeated twice in order to make sure that all exchangeable label was removed. The residue was ground up and suspended in 5 ml 0·5% methylcellulose.

The first dialysate was extracted three times with 50 ml methylene chloride and the aqueous phase was lyophilized as described above. The residue was taken up in 5 ml water and passed through a paper filter.

Isolation of DNA. DNA was isolated from the liver according to Markov & Ivanov (1974) with the modifications as described (Viviani & Lutz, 1978). DNA was dissolved in 0·014 M-sodium phosphate buffer, pH 6·8, and counted after the addition of 10 ml Insta-Gel (Packard Instruments, Downers Grove, IL, USA) in a liquid scintillation counter (Betaszint 5000, Laboratorium Prof. Dr. Berthold, Wildbad, Federal Republic of Germany). The limit of detection of radioactivity is given by the total variability of the background samples, and one standard deviation (SD) was determined to be 0·7 cpm. A limit of detection of 1·5 dpm (2 SD) was therefore adopted for the ^{14}C experiments and 3 dpm for the tritium label.

Experiment on [^{14}C]AFB_1. Two 280-g male rats were each given orally a dose of $3{\cdot}6 \times 10^7$ dpm (320 μg) [^{14}C]AFB_1/kg. After 6 hr, the high-molecular fraction was isolated from the combined livers as described above and administered in three portions of about 5·5 ml at hourly intervals to another rat, which was killed 10 hr after receiving the last portion, for isolation of its liver DNA. Numerical details are presented in Table 1.

Experiment on [^{3}H]AFB_1. A 288-g male rat received by oral gavage 9×10^8 dpm (12 μg) [^{3}H]AFB_1/kg, together with $1{\cdot}2 \times 10^9$ dpm tritiated water contaminant/kg. After 6 hr, the aflatoxin metabolites were isolated from the liver as described above, and the high-molecular derivatives were administered to one rat and the low-molecular metabolites to another rat. Liver DNA was isolated from these rats after 9 hr. Numerical details are given in Table 2.

Control experiment with tritiated water, HTO. One 290-g rat received by oral gavage $4{\cdot}2 \times 10^9$ dpm HTO/kg. After 6 hr, the macromolecules were isolated from the liver as described above and administered to another (330-g) rat ($5{\cdot}3 \times 10^4$ dpm/kg), from which 16·7 mg liver DNA was isolated after 9 hr. The gross radioactivity (22·7 cpm, 5·40 min counting time) was not significantly different from that of the control DNA (22·5 cpm).

Table 1. *Binding of macromolecule-bound [^{14}C]aflatoxin B_1 to rat-liver DNA 10 hr after its oral administration*

Parameter	Control rat	Treated rat
Body weight (g)	221	228
Radioactivity administered (dpm/kg)	0	$3{\cdot}6 \times 10^6$
Liver DNA		
Amount in scintillation vial (mg)	1·69	1·62
Gross activity (cpm; 240-min count)	23·6	22·9
Specific activity of DNA (dpm/mg)*	—	<0·9
DNA binding (dpm/mg DNA) per dose (dpm/kg)	—	$<2{\cdot}6 \times 10^{-7}$
Covalent binding index (CBI)†	—	<80‡

*Based on 1·5 dpm as limit of detection.

†CBI = (μmol chemical bound per mol DNA phosphate)/(mmol chemical administered per kg body weight).

‡CBI determined for aflatoxin B_1 under similar experimental conditions was 10,000.

Table 2. *Binding of macromolecule-bound [^3H]aflatoxin B_1 and water-soluble [^3H]aflatoxin conjugates to rat-liver DNA 9 hr after their oral administration*

Parameter	Control rat	Rat treated with: Macromol.-bound aflatoxin	Rat treated with: Water-soluble conjugates
Body weight (g)	310	320	415
Radioactivity administered (dpm/kg)	0	$2{\cdot}8 \times 10^7$	$8{\cdot}3 \times 10^5$
Liver DNA			
Amount in scintillation vial (mg)	11·9	14·6	11·4
Gross activity (cpm; 200-min count)	22·5	22·8	22·4
Specific activity of DNA (dpm/mg)*	—	<0·21	<0·26
DNA binding (dpm/mg DNA) per dose (dpm/kg)			
	—	$<7{\cdot}4 \times 10^{-9}$	$<3{\cdot}2 \times 10^{-7}$
Covalent binding index (CBI)†	—	<2·3‡	<98‡

*Based on 3 dpm as limit of detection.
†CBI = (μmol chemical bound per mol DNA phosphate)/(mmol chemical administered per kg body weight).
‡CBI determined for aflatoxin B_1 under similar experimental conditions was 10,000.

RESULTS

The fraction of aflatoxin radioactivity recovered from the rat liver 6 hr after a single dose of AFB_1 was found to be 9% in the experiment with the ^{14}C-label and 7% with the ^3H-label. Of this total radioactivity, 40% (^{14}C-label) and 46% (^3H-label) was bound to macromolecules, 2% (^3H-label) was recovered in the low-molecular fraction. The fraction of macromolecule-bound aflatoxin metabolites is of the same order as that found in the pig (Lüthy *et al.* 1980), and is in good agreement with earlier studies in the rat (Wogan, Edwards & Shank, 1967).

Tables 1 and 2 give data on the binding experiments and show that in no case was any radioactivity detectable on the liver DNA of the rats given the radiolabelled aflatoxin derivatives isolated from the livers of the sponsor rats. The lowest corresponding value for the CBI is easily calculated from these data if the limit of detection of radioactivity (1·5 dpm for ^{14}C, 3 dpm for tritium) is divided by the amount of DNA isolated and by the dose of radioactivity administered to the animal.

Macromolecule-bound aflatoxin derivatives

With the ^{14}C-label (Table 1), the limit of detection of binding of macromolecule-bound aflatoxin metabolites to rat liver DNA was found to be 80 in the CBI units (μmol chemical bound/mol nucleotides)/(mmol chemical administered/kg body weight). With the ^3H-label (Table 2), a larger amount of radioactivity was available so that a value of 2·3 resulted for the highest possible CBI for rat liver DNA. This figure is more than 4000 times below the CBI of AFB_1 itself.

Low-molecular aflatoxin conjugates

Only one experiment, with the tritium label (Table 2), was performed with this minor fraction isolated from the liver of a rat that had received AFB_1. The radioactivity that could have been expected from an experiment with ^{14}C would have been too low for a DNA binding experiment with a second rat. The limit of detection of a CBI of less than 100 shows that this fraction of the aflatoxin residues in rat liver is more than 100 times less active than AFB_1 itself with respect to DNA binding in the rat liver.

HTO control

Because of the contamination of the [^3H]AFB_1 with tritiated water a check was made on the extent to which HTO was incorporated into the macromolecular fraction used in the main experiment. The amount of tritium administered was about twice as much as that given in the [^3H]AFB_1 experiment, but only about 0·001% of the radioactivity administered was recovered on the macromolecules. Administration of this total macromolecular fraction to a second rat resulted in no detectable radioactivity on the liver DNA. These results show that the high percentage of HTO in the original ^3H-AFB_1 dose did not affect the interpretation of the results.

There is a possibility that macromolecule-bound aflatoxin metabolites lose their label during the isolation procedure and during the time between the administration of the radioactivity and its measurement on the DNA. A lack of any radioactivity on the DNA could then be due partly to a loss of the label. This possibility can be excluded, however, since it was shown by the repeated lyophilization steps, carried out at daily intervals, that less than 1% of the radioactivity on the macromolecule-bound aflatoxin metabolites was exchanged within 1 day.

DISCUSSION

CBIs for aflatoxins have so far been determined with AFB_1 by Campbell, Hayes & Newberne (1978), Croy, Essigmann, Reinhold & Wogan (1978), Garner & Wright (1975), Preston, Hayes & Campbell (1976) and Swenson, Lin, Miller & Miller (1977) and also in this laboratory (Lutz, 1979), figures between 10,000 and 30,000 having been reported for rat-liver DNA. AFB_2 has been investigated by Swenson *et al.* (1977), AFM_1 by Lutz (1979) and AFG_1 by Garner, Martin, Smith, Coles & Tolson (1979). The CBI of these three aflatoxins ranged between 500 and 4000 so that a good quantitative correlation with the observed carcinogenicity from long-term experiments was established. Some early experiments with AFB_1 and AFG_1, based upon tritiated aflatoxins, were performed by Lijinsky, Lee & Gallagher (1970), who obtained a CBI for AFB_1 much lower than those found in all the

other laboratories, possibly as a result of using an unstable tritium label.

Our results with aflatoxin residues isolated from rat liver reveal that neither macromolecule-bound metabolites nor low-molecular conjugates exhibit any DNA-binding ability. Macromolecule-bound AFB_1 residues could be at the worst 4000 times less hepatocarcinogenic than AFB_1 itself, while the conjugates are at least 100 times less active than AFB_1 with respect to covalent binding to rat-liver DNA.

A CBI describes the DNA damage to be expected after the administration of a unit dose. If the real amount of DNA-bound chemical from a specific dose has to be assessed, the actual dose has also to be taken into account. As a first approximation, a multiplication of the CBI with the dose might be performed, although this is only a valid procedure if a linear dose-binding relationship has been demonstrated over the complete dose range in question.

The actual exposure of humans to aflatoxin residues from pork liver can be estimated on the basis of the metabolism data presented by Lüthy *et al.* (1980), following the making of appropriate assumptions on the contamination of the pig diet with aflatoxins. It can then be deduced that pork liver in Switzerland contains less than 0·1 ppm macromolecule-bound aflatoxin metabolites, while the amount of water-soluble aflatoxin conjugates will be much lower even than that. In muscle, the main edible tissue, levels of both types of residue are again lower by several orders of magnitude.

If such low exposure is taken into account in assessing the carcinogenic risk of a population consuming pork liver or meat, the risk must be considered negligible when compared with that from the direct intake of aflatoxins from nuts, milk and other sources.

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EFFECTS OF MYCOTOXINS ON MIXED-FUNCTION OXIDASE AND ADENOSINE TRIPHOSPHATASE SYSTEMS IN NEONATAL RATS. I. AFLATOXIN B_1/RUBRATOXIN B

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Abstract—The effects of aflatoxin B_1 and/or rubratoxin B on hepatic monooxygenase and hepatic, brain and renal ATPase activities were examined in neonatal rats exposed to a single treatment of one or both toxins. Animals received orally 0·4 mg aflatoxin B_1/kg, 2 mg rubratoxin B/kg or the same doses of aflatoxin B_1 and rubratoxin B in combination, within 24 hr of birth, and were killed 12 days later. Given alone, rubratoxin B caused a statistically significant decrease in cytochrome *P*-450 content. Hepatic and renal oligomycin-sensitive ATPase activity was depressed by the aflatoxin B_1 and rubratoxin B combination but this effect appeared to be the result of the aflatoxin treatment. The only interaction observed after exposure of pups to the mycotoxin combination was a tendency towards a decrease in NADPH-dependent dehydrogenase.

INTRODUCTION

Mycotoxins, secondary fungal metabolites produced as contaminants of foodstuffs and feeds, can cause illness and death if ingested by man and animals (Kreshnamachari, Ramesh, Ghat & Tilak, 1975; Newberne, 1974). Contamination of agricultural products by more than one toxigenic fungus and/or mycotoxin has been implicated in mycotoxicoses such as toxic aleukia (Mayer, 1953), the haemorrhagic anaemia syndrome (Forgacs & Carll, 1962) and toxic hepatitis (Wilson & Hayes, 1972). Sippel, Burnside & Atwood (1953) described a disease in swine and cattle involving mouldy corn from which Burnside, Sippel, Forgacs, Carll, Atwood & Doll (1957) later isolated *Aspergillus flavus* (aflatoxin B_1) and *Penicillium rubrum* (rubratoxin B).

Potentiation of the lethal action of rubratoxin B, but not of the carcinogenicity of aflatoxin B_1, occurred in adult rats treated with rubratoxin and fed over the same period a diet containing aflatoxin (Wogan, Edwards & Newberne, 1971). In experiments in day-old rats using rubratoxin B and aflatoxin B_1, Hayes, Cain & Moore (1977) demonstrated not only that neonatal rats were more susceptible than adult animals to each toxin alone but that the effects of the toxin combination on mortality and body-weight gains were interactive when the toxins were administered together to day-old animals. Richard, Thurston & Graham (1974) also reported that a combination of aflatoxin B_1 and rubratoxin B given to guinea-pigs for 3 wk resulted in more deaths than occurred with rubratoxin given alone.

In vivo inhibition of both ATPase and mixed-function oxidase systems occurred in adult male mice exposed to rubratoxin B (Desaiah, Hayes & Ho, 1977; Siraj & Hayes, 1979). The hepatic drug-metabolizing enzyme system was also inhibited by aflatoxin B_1 in rats (Gurtoo, Campbell, Webb & Plowman, 1968), possibly because of its inhibitory effect on protein synthesis (Smith, 1963). Although aflatoxin B_1 had limited effects on ATPase (Desaiah, Phillips, Hayes & Ho, 1979), transport Na^+/K^+ ATPase activities of brain, liver and kidney were inhibited by rubratoxin B (Desaiah *et al.* 1977). The parent rubratoxin molecule was a potent *in vitro* inhibitor of ATPase but alteration of either the α,β-conjugated γ-lactone or the maleic anhydride moieties of rubratoxin to the corresponding dihydro, lactol or open-ring carboxylic acid analogues decreased the inhibition (Phillips & Hayes, 1979).

Since aflatoxin B_1 and rubratoxin B had been shown to affect drug-metabolizing enzymes and ATPase *in vivo*, since multiple exposure to mycotoxins has been reported and since mycotoxins are foodborne and therefore liable to affect any age group, the susceptibility of the rat neonate to aflatoxin B_1 and rubratoxin B either singly or in combination was examined using transport ATPase and hepatic-drug metabolism as indices of toxicity.

EXPERIMENTAL

Chemicals. Aflatoxin B_1 was purchased from Calbiochem (La Jolla, CA). Rubratoxin B was prepared according to the procedure of Hayes & Wilson (1968). Purity (99%) of both compounds was confirmed by melting point, thin-layer and high-pressure liquid chromatography, and infra-red and mass spectral analyses. The mycotoxins were stored in the dark and freshly prepared in propylene glycol before use. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Treatment. Offspring of female Sprague-Dawley rats (Charles River, Wilmington, MA) obtained from our colony were pooled within 24 hr of parturition to form four groups, each containing four litters of 10–12 pups/litter. Pups were treated by gastric intubation (PE 10 tubing) on day 1 of age with an LD_{25} dose of

either aflatoxin B_1 (0·4 mg/kg) or rubratoxin B (2·0 mg/kg), an aflatoxin–rubratoxin combination (0·4 and 2·0 mg/kg, respectively) or the solvent vehicle (propylene glycol) and were then returned to the dam. Pups were killed on day 13. Body weights were recorded before and on day 13 after treatment. Throughout the experiment, litters were maintained on corncob bedding in individual cages under a 12-hr light/dark cycle, in temperature-controlled facilities (72 ± 2°F) away from known hepatic-enzyme inducers. Standard laboratory chow and water were freely available to the dams at all times.

Sample preparation. Animals were decapitated and the brain, kidneys and liver were quickly removed. Livers from pups in the same litter were pooled and blotted and a random portion (2–3 mg) was removed for ATPase analysis. Brains and kidneys from pups in the same litter were also pooled. All three of these samples were placed in ice-cold 0·32 M-sucrose solution (pH 7·5), containing 1·0 mM-EDTA and 10 mM-imidazole for homogenization and tissue fractionation (Phillips & Hayes, 1977).

The rest of the pooled liver from each group was weighed and homogenized in ice-cold isotonic KCl to form a single sample which was used to prepare microsomal fractions in which drug-metabolizing enzyme activity and cytochrome *P*-450 content were determined.

Three treatment groups and a control group, each containing tissues (brain, liver or kidney) from at least ten pups, were processed.

Studies of monooxygenase system. Hepatic microsomes were isolated and drug-metabolizing enzymes were analysed using procedures detailed earlier (Siraj & Hayes, 1979). The effects of the mycotoxins, singly and in combination, on hexobarbital hydroxylase (Cooper & Brodie, 1955), aniline hydroxylase (Kato & Gillette, 1965), the production of formaldehyde by the demethylation of ethylmorphine (Nash, 1953), the activities of aminopyrine *N*-demethylase (La Du, Gaudette, Trousef & Brodie, 1955), of NADPH-dependent dehydrogenase (Gillette, Brodie & La Du, 1957), and of NADPH-cytochrome *c* reductase (Williams & Kamin, 1962), and the cytochrome *P*-450 content (Omura & Sato, 1964) were determined. Enzyme activities and cytochrome *P*-450 content were calculated per mg microsomal protein and are presented as nmol product/mg protein/30 min.

Studies of ATPase system. ATPase analyses were carried out as previously described (Phillips & Hayes, 1977). Regression analysis of standard inorganic phosphate and protein curves was used to calculate specific activities (μmol P_i/mg protein/hr). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as the standard.

Expression of results. Data were subjected to two-way analysis of variance, and differences with $P < 0·05$ were considered significant.

RESULTS

There was a significant reduction (25%) in average weight gain over the 12-day period in pups given the combined aflatoxin–rubratoxin treatment, whereas the mean weight gains for pups exposed to only aflatoxin or rubratoxin were not below that of the controls. No significant differences were observed in liver/body-weight ratios between any of the groups.

Effects on monooxygenases

The effects of aflatoxin B_1 and rubratoxin B, singly or in combination, on the hepatic mixed-function oxidase system in rat neonates are presented in Table 1. In comparison with control animals, a statistically significant decrease of 26% was observed in the cytochrome *P*-450 content in rubratoxin-treated animals. Aflatoxin B_1 and the mycotoxin combination caused decreases in the cytochrome *P*-450 content to 86 and 71% of control values, respectively. The dehydrogenase activity was not affected in animals exposed to rubratoxin B or aflatoxin B_1 but was inhibited by 19% in the combination group. The NADPH-cytochrome *c* reductase activity was unaffected by any of the treatments.

Aniline hydroxylase activity and demethylation of aminopyrine and ethylmorphine were unaffected by the mycotoxins, separately or in combination (Table 1). Rubratoxin B and the mycotoxin combination decreased hexobarbital hydroxylase activity to 59 and 71% of the control values, respectively. Hexobarbital oxidation was not affected by aflatoxin B_1. The microsomal protein values were unaffected by exposure of the animals to rubratoxin B and/or aflatoxin B_1 (Table 1).

Effects on ATPase

The effects of aflatoxin B_1 or rubratoxin B either singly or in combination on mitochondrial ATPase activities from brain, liver and kidney are summarized in Table 2. Brain ATPase activities were not affected in the mitochondrial fraction regardless of treatment. However, significant ($P < 0·05$) inhibition of hepatic oligomycin-sensitive ATPase occurred in groups treated with aflatoxin B_1 or with the aflatoxin–rubratoxin combination (31·4 and 28·4%, respectively). Renal oligomycin-sensitive ATPase was inhibited significantly by aflatoxin B_1 and by the toxin combination (28·6 and 29·7%, respectively). (Na^+-K^+)-activated ATPase was not affected. Rubratoxin alone had no effect on either liver or kidney ATPase activity.

The microsomal ATPase activities were not affected by aflatoxin, rubratoxin or the mycotoxin-combination in either brain, liver or kidney (data not shown).

DISCUSSION

The 12-day oral LD_{25} values determined for aflatoxin B_1 and rubratoxin B in our rats were 0·4 and 2·0 mg/kg, respectively (unpublished data). These values were similar to those reported by Wogan *et al.* (1971) and Hayes *et al.* (1977). The results reported here demonstrate that the mitochondrial ATPase system, certain hepatic drug-metabolizing enzymes and the hepatic cytochrome *P*-450 content were impaired in 12-day-old rats that had been treated on day 1 after birth with a combined dose of aflatoxin B_1 and rubratoxin B (the LD_{25} of each toxin), and that the same enzymes were affected also by the LD_{25} of one or other of the mycotoxins given alone. Hepatic and

Table 1. *Effects of aflatoxin B_1 and/or rubratoxin B on hepatic drug-metabolizing enzymes, cytochrome* P-*450 content and the oxidation of selected substrates in neonatal rats*

	Values for groups of rats treated with						
	Propylene glycol (control)	Rubratoxin B (2·0 mg/kg)		Aflatoxin B_1 (0·4 mg/kg)		Rubratoxin + aflatoxin (2·0 mg/kg) (0·4 mg/kg)	
Parameter†	Mean	Mean	%	Mean	%	Mean	%
Protein (mg/g wet tissue)	23·9 ± 0·8	24·9 ± 1·4	104	26·1 ± 0·8	109	24·9 ± 1·8	104
Cytochrome *P*-450 (nmol/mg mpr)	1·14 ± 0·04	0·84 ± 0·05*	74	0·98 ± 0·10	86	0·81 ± 0·02*	71
NADPH-dependent dehydrogenase‡	40·2 ± 4·9	38·7 ± 4·7	96	48·1 ± 6·4	119	32·7 ± 3·3	81
NADPH-cytochrome *c* reductase‡	188·1 ± 23·4	172·4 ± 5·4	92	175·2 ± 16·3	93	181·6 ± 16·2	97
Hexobarbital hydroxylase§	14·8 ± 1·2	8·7 ± 3·2*	59	12·6 ± 2·0	85	10·6 ± 1·75*	71
Aniline hydroxylase§	8·45 ± 2·3	8·3 ± 2·3	98	7·6 ± 2·0	90	10·3 ± 2·3	122
Aminopyrine *N*-demethylase§	8·7 ± 0·3	8·0 ± 0·6	92	7·6 ± 0·2	88	8·8 ± 0·2	101
Ethylmorphine *N*-demethylase§	22·0 ± 2·0	18·9 ± 0·9	86	17·9 ± 2·0	81	19·2 ± 2·2	87

mpr = Microsomal protein

†Determined on hepatic microsomes isolated from 13-day-old rats that had been treated with the mycotoxin(s) by gavage within 24 hr of birth.

‡Expressed as nmol/mg mpr/3 min.

§Expressed as nmol/mg mpr/30 min.

Results are expressed as means ± SEM for groups of 10–12 pups and then as a percentage of the control value. Means marked with an asterisk differ significantly from the control figure: (*$P \leqslant 0{\cdot}05$ by two-way analysis of variance).

Table 2. *Effects of aflatoxin B_1 and/or rubratoxin B on mitochondrial ATPase activities from brain, liver and kidneys of neonatal rats*

Enzyme		Specific activity (μmol P_i/mg protein/hr) in mitochondrial fractions from rats treated with: Propylene glycol (control) Mean	Rubratoxin B (2·0 mg/kg) Mean	Rubratoxin B (2·0 mg/kg) %	Aflatoxin B_1 (0·4 mg/kg) Mean	Aflatoxin B_1 (0·4 mg/kg) %	Rubratoxin + aflatoxin (2·0 mg/kg) (0·4 mg/kg) Mean	Rubratoxin + aflatoxin (2·0 mg/kg) (0·4 mg/kg) %
				Brain				
Na^+-K^+ ATPase		29·9 ± 2·0	26·8 ± 3·1	90	31·6 ± 1·6	106	32·6 ± 3·1	109
Mg^{++} ATPase:	OS	15·5 ± 2·5	16·5 ± 5·0	106	16·3 ± 1·2	105	17·3 ± 1·3	112
	OI	13·6 ± 2·3	12·6 ± 1·3	93	15·8 ± 1·2	116	10·3 ± 1·0	76
				Liver				
Na^+-K^+ ATPase		—	—	—	—		—	
Mg^{++} ATPase:	OS	20·4 ± 1·9	18·8 ± 1·0	92	14·0 ± 1·3*	69	14·6 ± 1·1*	72
	OI	17·5 ± 2·2	20·2 ± 1·9	115	13·6 ± 2·5	78	15·5 ± 1·7	89
				Kidney				
Na^+-K^+ ATPase		21·3 ± 3·0	19·5 ± 1·4	92	20·2 ± 2·1	95	14·7 ± 2·0	69
Mg^{++} ATPase:	OS	18·5 ± 1·15	18·3 ± 3·0	99	13·2 ± 2·0*	71	13·0 ± 2·0*	70
	OI	13·6 ± 2·3	12·4 ± 3·1	91	12·2 ± 0·8	90	13·3 ± 0·6	98

OS = Oligomycin sensitive OI = Oligomycin insensitive

Results are expressed as means ± SEM for four independent replicate studies, each assayed in triplicate, and then as a percentage of the control value. Means marked with an asterisk differ significantly from the control figure: *$P \leq 0{\cdot}05$ by two-way analysis of variance.

renal mitochondrial oligomycin-sensitive ATPases were inhibited by aflatoxin B_1 and the mycotoxin-combination but not by rubratoxin B, suggesting that this effect was due to aflatoxin B_1 alone. We have previously demonstrated *in vitro* the inhibition of these enzymes by aflatoxin B_1 in the adult rat (Desaiah *et al.* 1979). The failure to detect an effect on the microsomal ATPase may be because the toxin was removed during the extensive washing and fractionation procedure (Phillips, Hayes, Ho & Desai, 1978).

Interaction between the two mycotoxins as well as the effects of a single toxin was observed with the hepatic drug-metabolizing enzymes and cytochrome *P*-450 system. The effect of the LD_{25} of rubratoxin on the hepatic microsomal mixed-function oxidase system in rat neonates was more pronounced than that induced by aflatoxin B_1. Decreases in cytochrome *P*-450 content and hexobarbital-hydroxylase activity were observed but only in neonates exposed to rubratoxin B. Although our values for the cytochrome *P*-450 content for 12-day-old rats were higher than those reported earlier (Iba, Soyka & Schulman, 1977), the significant decrease in cytochrome *P*-450 content by rubratoxin B was in agreement with our earlier finding of the inhibitory effect of this toxin on the hepatic mixed-function oxidase system in adult male mice (Siraj & Hayes, 1979). While the effects of aflatoxin B_1 or the mycotoxin combination on most of the liver enzymes measured were not significant, there was a tendency in several cases towards an inhibitory effect when rubratoxin B and aflatoxin B_1 were given together. The decrease in hexobarbital-hydroxylase activity and in cytochrome *P*-450 content in rat neonates exposed to the two toxins suggested, however, that these effects were due to rubratoxin B alone. A tendency towards potentiation of the effect of rubratoxin B by aflatoxin B_1 was observed only in the case of NADPH-dependent dehydrogenase. Overall, however, our results suggested that the effect of rubratoxin B on the hepatic microsomal drug-metabolizing enzyme system in neonatal rats was not enhanced by aflatoxin B_1.

In general it has been shown that although one toxin potentiated the lethal effect of the other toxin, the neonatal rat was more susceptible than the adult animal to the acute effects of aflatoxin B_1 and of rubratoxin B. Results from this study also suggest that interaction of the two toxins in respect of their effects on ATPase or hepatic drug-metabolizing enzymes was limited or absent. However, significant inhibition of mitochondrial ATPase and the hepatic drug-metabolizing enzyme system was demonstrated following exposure to a single toxin (aflatoxin B_1 and rubratoxin B, respectively).

The increased susceptibility of neonates to these mycotoxins may be due in part to the immaturity of the excretory mechanisms, including transport Na^+-K^+ ATPase, which are not fully developed by day 13 in the rat (Klaassen, 1972; Samson, Dick & Balfour, 1964). Inhibition of the "already impaired" excretory system by mycotoxins such as aflatoxin B_1 and rubratoxin B may overburden the system. Thus, toxic effects are manifested in the neonate at dose levels lower than those required to produce similar effects in adult animals.

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EFFECTS OF FEEDING T-2 TOXIN TO RATS AND MONKEYS

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Abstract—Feeding a diet containing mouldy sorghum that supplied 25 ppm T-2 toxin per day to rats for 16 wk produced marked growth depression and loss of body fur. No other clinical signs or histopathological changes were observed. Semi-purified T-2 toxin was obtained from rice culture by a series of extraction procedures and was free of other trichothecenes. The toxin was administered by stomach tube to rhesus monkeys at levels equivalent to 1 or 0·5 mg of the pure compound/kg body weight/day for 15 days, and signs somewhat similar to those of alimentary toxic aleukia in man were observed. Male monkeys were more susceptible to the toxic effects of T-2 toxin than the females and all the male animals developed severe leucocytopenia and died between days 8 and 15 of treatment. The lungs of the dead animals showed grey hepatization. The spleen and lymph nodes showed the presence of atrophic follicles, indicating the involvement of the immune system. At an oral dose equivalent to 0·1 mg pure T-2 toxin/kg body weight/day both male and female monkeys developed leucocytopenia and mild anaemia after 15 days of treatment.

INTRODUCTION

Trichothecenes, a group of chemical compounds that possess a common structural nucleus, are produced in laboratory cultures by species of Fusarium, Trichothecium, Stachybotrys and some other genera of fungi (Smalley & Strong, 1974). Some of these toxic metabolites have been isolated from food grains naturally contaminated with species of Fusarium and Stachybotrys and have been shown to be associated with outbreaks of disease in farm animals and humans (Joffe, 1974; Rodricks & Eppley, 1974; Saito & Ohtsubo, 1974). T-2 toxin, a prominent member of the trichothecene group has been frequently analysed and reported in literature (Rukmini & Bhat, 1978; Smalley, Marasus, Strong, Bamburg, Nichols & Kosuri, 1970). It is now fairly well established that the problem of toxicosis associated with trichothecenes is not limited to countries with temperate climates. Observations on the natural occurrence of trichothecenes in a number of agricultural products and evidence suggesting that alimentary toxic aleukia (ATA) in man has been caused by T-2 toxin, emphasize the importance of these toxins as hazards to human and animal health.

The predominant clinical features of ATA in man are inflammation of the skin and mucous membrane, vomiting, leucocytopenia and bone-marrow depression (Mayer, 1953). The adverse effects of trichothecenes in cattle and swine include emesis, anorexia and depression of growth, followed by death in acute cases (Ueno, 1977a). Low egg production (Wyatt, Doerr, Hamilton & Burmeister, 1975), neural disorders, leucocytopenia (Wyatt, Colwell, Hamilton & Burmeister, 1973) and abnormal reproductive performance were observed in chickens (Chi, Mirocha, Kurtz, Weaver, Bates & Shimoda, 1977). Sato, Ueno & Enomoto (1975) observed marked leucocytopenia in cats given T-2 toxin isolated from *F. solani*. There have so far been no reports of the effects of T-2 toxin in primates. Also there appears to be no experimental model for studying the biological effects of the toxin in animals using naturally infected food grains. The results of studies in which mouldy sorghum was fed to rats and semi-purified T-2 toxin was administered to monkeys are presented in this paper.

EXPERIMENTAL

Materials. Sorghum that was naturally contaminated with *Fusarium incarnatum* was used in the rat feeding experiments. Chemical and spectral analysis (Rukmini & Bhat, 1978) established that it contained T-2 toxin. Quantitative analysis (Ikediobi, Hsu, Bamburg & Strong, 1971) using a reference standard of T-2 toxin (kindly supplied by Dr. Y. Ueno of Tokyo University of Sciences, Japan) showed that the sorghum contained 24·5 mg T-2/kg. For the experiments using monkeys, *F. incarnatum* that had been isolated from sorghum was grown on healthy grains of rice under optimal conditions and semi-purified T-2 toxin was isolated by several extraction procedures as described previously (Rukmini & Bhat, 1978). The final chloroform–methanol (93:7, v/v) extract contained only T-2 toxin, and no other trichothecenes were present. The solvents were removed by evaporation under reduced pressure and the residual semi-purified toxin was administered to the monkeys.

Studies on rats. Weanling Wistar albino rats, reared at the Institute, were divided into two groups of 12 animals (six males and six females). They were fed diets containing either 90% uncontaminated sorghum or 90% mouldy sorghum. The diet containing mouldy sorghum supplied 25 ppm T-2 toxin per day. The diet also contained 5% peanut oil, 4% mineral mixture (Association of Official Analytical Chemists, 1965) and 1% vitamin mixture (National Academy of Sciences–National Research Council, 1963). In terms of protein content and amino acid composition the

Table 1. *Haematological data for monkeys given semi-purified T-2 toxin or uncontaminated sorghum extract by stomach tube*

Treatment group	No. and sex of monkeys	Values for samples taken on day		
		0	7	15
		Haemoglobin level (g/100 ml whole blood)		
Control	3M + 3F	13·60 (12·5–14·1)	ND	13·06 (12·5–13·5)
I	3M + 2F	13·72 (13·0–14·5)	13·02 (12·0–13·6)	12·43 (12·2–12·9)*
II	2M + 2F	15·35 (14·1–16·3)	12·62 (12·5–13·3)	12·40 (12·1–12·8)
		White blood cell count ($\times 10^3$/mm^3)		
Control	3M + 3F	11·40 (10·08–12·4)	ND	11·40 (10·28–12·8)
I	3M + 2F	14·58 (12·2–16·0)	10·72 (9·4–12·2)	8·50 (8·0–9·0)*
II	2M + 2F	12·32 (11·90–12·85)	12·10 (9·3–13·6)	8·10 (7·6–8·4)
		Platelet count ($\times 10^5$/mm^3)		
Control	3M + 3F	4·50 (3·8–4·7)	ND	4·70 (3·0–5·8)
I	3M + 2F	4·46 (4·1–5·2)	3·87 (3·0–4·5)	3·80 (3·6–4·0)*
II	2M + 2F	4·05 (3·7–4·7)	4·15 (3·8–4·8)	3·95 (3·9–4·0)

ND = Not determined

*Results are for the two female monkeys; the males died between days 8 and 15.

Values are means, and in brackets the range, for the number of animals indicated.

The control group was given extract from uncontaminated sorghum. Group I was given semi-purified T-2 toxin at a level equivalent to 1 mg pure T-2 toxin/kg body weight/day on days 1–4 and at the equivalent of 0·5 mg T-2 toxin/kg/day on days 5–15. Group II was given the equivalent of 0·1 mg pure T-2 toxin/kg body weight/day. The two female monkeys in Group II had previously been treated in Group I.

mouldy sorghum was somewhat inferior to the good sorghum. Feeding was continued for 16 wk and body weights and food intake were recorded at weekly intervals. Signs of toxicity such as diarrhoea, vomiting, redness of the skin and hair loss were recorded. After 16 wk the animals were killed and the organs were examined histopathologically by conventional methods.

Studies on monkeys. Adult rhesus monkeys weighing 2–3 kg were divided into two groups. The control group consisted of three males and three females, and in the treated group there were three males and two females. The monkeys were fed a stock diet that contained adequate amounts of all essential nutrients. The animals in the treated group were given daily, by stomach tube, T-2 toxin in 20 ml milk. The control animals were dosed with milk containing an extract of uncontaminated sorghum. Initially all the animals in the treated group received a daily dose equivalent to 1 mg of the pure T-2 toxin/kg body weight. However, on the second day, the males started to vomit within 3 hr of dosing and became extremely weak. Therefore the toxin dose was reduced to the equivalent of 0·5 mg pure compound/kg body weight/day from day 5–15. All three male monkeys given T-2 toxin died between days 8 and 15. Subsequently, after 30 days recovery, the two treated female monkeys and two additional male monkeys were given 0·1 mg T-2 toxin/kg body weight/day for 15 days.

Blood samples were taken from all the animals at the start of the experiment and then at weekly intervals, and haemoglobin content, numbers of red and white blood cells and packed cell volume were determined by standard methods. Autopsies were carried out on the males that died between days 8 and 15 and on one male and one femal monkey from the control group. Tissues and organs such as the lymph nodes, bone marrow, thymus, spleen and intestines were processed for histopathological examination. These tissues were fixed in 10% neutral buffered formalin and stained with haemotoxylin and eosin, Masson's trichrome stain and Gomori's stain.

RESULTS AND DISCUSSION

Studies on rats

There were no apparent differences in food intake between the two groups. However, the animals receiving T-2 toxin in mouldy sorghum gained significantly less weight than the controls, the average difference in body weights after 16 wk being nearly 30 g. Apart from loss of fur, no abnormalities were seen in the animals fed mouldy sorghum and histopathological examination of the tissues revealed no significant abnormalities.

These observations were similar to those in earlier studies. Albino Holtzman rats fed T-2 toxin at 5 ppm in the diet for 3 wk showed slight growth depression while in those fed 15 ppm considerable weight reduction was observed after 19 days. The rats showed severe inflammation around the nose and mouth (Marasas, Bamburg, Smalley, Strong, Ragland & Degurse, 1969). However, in the same study no ill-effects were observed in rats fed 15 ppm T-2 toxin for 19 days and then untreated feed or feed containing 10 ppm T-2 toxin in 4-wk cycles for 8 months. Relatively small doses of crude extracts from cultures of *Fusarium poae* or *F. sporotrichoides* given by stomach tube had local cytotoxic effects that were followed by regeneration and basal cell hyperplasia of the squamous epithelium of the oesophagus and stomach (Schoental & Joffe, 1974). Ueno (1977b) reported inflammation near the nose and mouth of rats fed 15 ppm T-2 toxin in the diet for 4 wk. No specific toxic effects were seen in the present study, in which about 25 ppm T-2 toxin was fed in the form of mouldy sorghum, and we therefore consider that per-

haps the rat is not a suitable model for the routine screening of food materials likely to be infected with Fusarium species.

Studies on monkeys

Signs of toxicity were observed in all the monkeys given 1 mg T-2 toxin/kg/day. These included vomiting, apathy and weakness of the lower limbs. However, only the male monkeys developed petechial haemorrhages on the face. Diarrhoea occurred in some animals. The toxic effects were more pronounced in males than in females. In males after days 4–5 of treatment the respiratory rate increased and later respiration became shallow and difficult. All three male monkeys had died by day 15 and their deaths appeared to be due to respiratory failure. At autopsy, congestion of the lungs and grey hepatization were observed. There were accumulations of fluid in the visceral cavities. The ratios of splenic weight to body weight in the three male monkeys that died were lower than those in the controls. In one treated monkey, the spleen and lymph nodes showed atrophic follicles.

When the toxin was administered at a high level (1 or 0·5 mg/kg body weight/day) severe leucocytopenia was observed in all the treated animals at the end of the first week (Table 1). Leucocyte counts dropped from an initial value of $14{\cdot}58 \times 10^3/mm^3$ to $10{\cdot}72 \times 10^3/mm^3$ on day 8, and to $8{\cdot}50 \times 10^3/mm^3$ on day 15. The decrease was due mainly to a reduction in the number of polymorphonuclear leucocytes, there being no change in the number of lymphocytes. Haemoglobin levels in the surviving treated monkeys fell from an initial value of 13·72 g/dl whole blood to 12·43 g/dl whole blood on day 15 while packed cell volume decreased from 40% on day 7 to 34% after 15 days treatment. The platelet count decreased from $4{\cdot}46 \times 10^5/mm^3$ on day 0 to $3{\cdot}80 \times 10^5/mm^3$ on day 15.

In the second phase of the study, when a smaller dose of toxin (0·1 mg/kg body weight/day) was used, leucocytopenia developed slowly and was marked only on day 15. Haemoglobin levels fell from an initial level of 15·3 to 12·4 g/100 ml. These data suggest that in monkeys, toxic effects can be induced by T-2 toxin given orally at levels of 0·1 mg/kg body weight/day. They also show that male monkeys are more susceptible to the toxic effects of T-2 toxin than females. Haematological and histopathological findings indicate that one of the effects of the toxin is immune suppression. Further studies are planned to evaluate a safe level of T-2 toxin by feeding the toxin to monkeys. It is also proposed to study in greater detail the immunological effects of T-2 toxin in experimental animals.

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INVESTIGATIONS ON THE TOXICOLOGY AND SAFETY OF ALGAL DIETS IN ALBINO RATS

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Abstract—The green alga *Scenedesmus acutus* was cultivated in clean water on a pilot-plant scale, and after harvest it was drum dried at 120°C for 5–8 sec. The drum-dried alga was fed *ad lib.* to weanling albino rats as the sole protein source at protein levels of 10 or 15% in the diet for 12 wk. Control rats were given diets containing 10% casein. Treatment at either dietary level did not result in any harmful histological or haematological effects. Urine analyses did not indicate any abnormalities in any of the treated groups. Liver cholesterol levels were lower in animals given the algal diets than in the controls while serum cholesterol levels were similar in the treated and control groups. The activities of the hepatic enzymes succinic dehydrogenase and alanine aminotransferase in the treated groups were similar to those in the controls. Gel electrophoresis did not show any major differences between the serum proteins of the test animals and those of the controls.

INTRODUCTION

The shortage of food in general and of proteins in particular needs no emphasis. To alleviate this problem, the use of several unconventional proteins including oil-seed proteins, fish- and leaf-protein concentrates and single-cell protein (SCP) has been suggested (Spansley, Halliday & Elizabeth, 1972; Waslien, 1975). SCP has been considered as a protein source only fairly recently and world-wide attention is being given to developing suitable technology to enable it to be used in feed and food.

Scenedesmus acutus, a unicellular green alga, is being studied as a potential source of SCP at the Central Food Technological Research Institute, Mysore. The alga produced from pilot-plant cultivation and processed by drum drying has already been evaluated for its protein quality (Becker, Venkataraman & Khanum, 1976a,b), and the protein efficiency ratio, net protein utilization, digestibility coefficient and biological value of the drum-dried alga have been found to be promising.

Guidelines for short-term and long-term feeding tests on algae and other sources of SCP have been outlined by the Protein–Calorie Advisory Group (PAG) of the United Nations (1970a,b). These recommended tests are being carried out to establish the safety of *Scenedesmus acutus*. In this preliminary study, the effects of feeding alga-containing diets to albino rats for 12 wk are critically evaluated. These early results form part of more rigorous tests but they have already provided some necessary basic information on the safety of algae as potential human food.

EXPERIMENTAL

Preparation of the alga. Scenedesmus acutus var. *alternans* (*Sc. obliquus* (TURP.) Krüger; strain 276–3a) was obtained from the culture collection of the University of Göttingen. Mass outdoor cultivation of this alga was done in plastic tanks. Suitable fertilizers were added and the cultures were aerated with 1% CO_2 in air to increase the yield. At its optimal growth rate, the alga was harvested by centrifugation and dried on a drum drier at 120 C for about 5–8 sec. The details of the cultivation and processing methods have been reported elsewhere (Venkataraman, Becker & Shamala, 1977).

Animals and diets. The compositions of the control and algal diets are shown in Table 1. In the test diets the alga was the sole source of protein. Casein was the protein source in the control diet. Nitrogen was estimated by the macro-Kjeldahl method and the protein contents of the feeds were calculated as N × 6·25, following the Indian Standard Method for the determination of protein efficiency ratio (Indian Standards Institution, 1975). Male Wistar weanling rats weigh-

Table 1. *Composition of experimental diets*

Component	Level of component (g/kg diet) in		
	Control diet	Algal diet I	Algal diet II
Casein	122*	—	—
Algae	—	240*	360*
Sugar	100	100	100
Oil	90	90	90
Mineral mixture†	40	40	40
Vitamin mixture‡	10	10	10
Corn starch	638	520	400

*The protein contents of the control, algal I and algal II diets were 10, 10 and 15%, respectively.

†The mineral mixture contained (g/kg mixture): KH_2PO_4, 389; $CaCO_3$, 381·4; NaCl, 139·3; $MgSO_4$, 57·3; $FeSO_4.7H_2O$, 27; $MnSO_4.H_2O$, 4·01; KI, 0·79; $ZnSO_4.7H_2O$, 0·548; $CuSO_4.5H_2O$, 0·477; $CoCl_2$, 0·23.

‡The vitaminized starch mixture contained (mg/g starch): choline, 200; inositol, 25; *p*-aminobenzoic acid, 10; calcium pantothenate, 4; niacin, 4; riboflavin, 1; vitamin K, 0·5; thiamine, 0·5; pyridoxine, 0·4; folic acid, 0·2; biotin, 0·02; vitamin B_{12}, 0·002.

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ing 40–48 g were randomly divided into three groups of eight. The rats were caged individually, and food and water were given *ad lib.* The animals were given the test diets for 12 wk, during which individual body weights were recorded weekly and food consumption was measured daily. Before the rats were killed, urine was collected for analysis. Blood was collected for haematological tests and for estimations of serum cholesterol and proteins.

Histopathological studies. The liver, kidneys, heart, lungs, brain, testes, spleen and thyroid, pituitary and adrenal glands of each rat were weighed, fixed in formalin, sectioned and stained with haematoxylin and eosin for histological examination. Frozen sections of the liver were stained with Sudan IV to study fatty infiltration.

Haematological assays. Haematological data such as haemoglobin content, red and white blood cell counts, differential count and packed cell volume were estimated by standard clinical procedures (Kolmer & Boerner, 1945).

Urine analyses. Pooled samples of urine taken at the end of the study from four rats in each dietary group were used for the analyses. Using standard clinical procedures (Kolmer & Boerner, 1945) the urinary pH and glucose and protein levels were determined and microscopic examination of the urinary sediment was carried out.

Determinations of serum and liver cholesterol. The lipids were extracted from serum and liver with alcohol-acetone (1:1, v/v) at 70°C for 10 min. The extract was made up to a defined volume with alcohol and the supernatant was used for cholesterol estimations. Total cholesterol was estimated by the method of Sackett (1925). A known volume of the extract was evaporated and the residue was taken up in chloroform. The colour was developed with a mixture of acetic anhydride–sulphuric acid (10:1, v/v) and absorbance was measured at 660 nm. The free cholesterol was precipated from the extract as digitonide and was dissolved in glacial acetic acid. Esterified cholesterol was estimated as the difference between total and free cholesterol levels. Polarizing microscopy was used to look for cholesterol deposition in the liver.

Studies of liver biochemistry. The nitrogen content of the liver was estimated by the micro-Kjeldahl method and the lipid content was determined by Soxhlet extraction. A 1% liver homogenate in 0·25 M-sucrose was prepared and succinic dehydrogenase activity was assayed by the method of Kun & Abood (1949). The assay mixture, containing 1·0 ml liver homogenate, 0·5 ml phosphate buffer, 0·5 ml 0·2 M-sodium succinate and 1·0 ml 0·1% triphenyltetrazolium chloride (TTC) was incubated at 38°C for 30 min. The reaction was stopped by addition of 7·0 ml acetone. After centrifugation the precipitate was discarded and the absorbance of the supernatant was read at 420 nm. Suitable blanks and standards were prepared under identical conditions. The enzyme activity was expressed as μg TTC reduced/10 min/mg fresh liver.

Alanine aminotransferase activity was assayed using a 10% liver homogenate in 0·1 M-phosphate buffer by the method of Tonhazy, White & Umbreit (1950). Sodium pyruvate was used as a standard. The absorbance of the final alcoholic KOH solution was read at 550 nm. The enzyme activity was expressed as mg pyruvate released/g fresh liver/10 min.

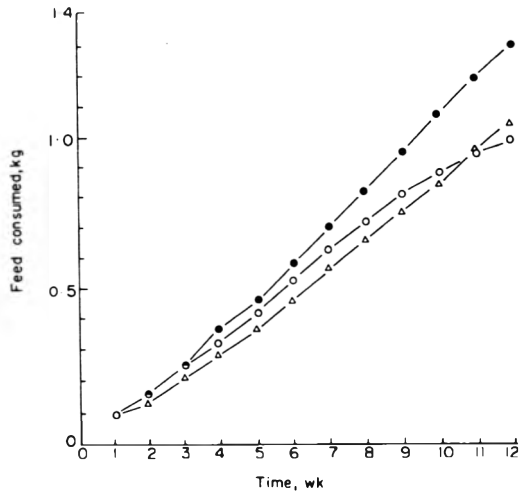

Fig. 1. Feed consumption by rats given diets containing 10% casein (—○—). 10% algal protein (—△—) or 15% algal protein (—●—) for 12 wk. Results are means for groups of eight rats.

Determination of serum proteins. Serum was prepared by clotting and centrifuging whole blood. The protein content of the serum was estimated by the biuret method (Gornall, Bardawill & David, 1949). Serum proteins were characterized by gel electrophoresis under the following conditions. Columns of 10% polyacrylamide gel were used and 50-mg aliquots of serum proteins were applied to the column. Spacer gel of large pore size was used as recommended by Davis (1964). Electrophoresis was conducted in 0·025 M-tris–glycine buffer at pH 8·3. A constant current of 3 mA per tube was applied for 90 min. The proteins were stained with 0·5% amido black in 7·5% acetic acid, and destaining was carried out in 7·5% acetic acid by diffusion. The band intensity was measured at 620 nm using a Chromoscan Microdensitometer (Chromoscan, Joyce-Loebel Ltd., Gateshead, England).

RESULTS

Food consumption and body and organ weights

The food consumption and the weight gain of the rats during the 12-wk feeding period are shown in Figs 1 and 2. The rats given the diet containing the 15% algal protein consumed more feed than those given the 10% algal protein- and casein-containing diets. The mean final body weights of the groups given the control, 10% algal protein and 15% algal protein diets were 189·75 (SD = 5·87), 207·13 (SD = 6·89) and 252·38 (SD = 9·83) g, respectively, and the differences between the three groups were significant ($P < 0·01$). The absolute weights of the various organs followed the same trend as the body weights, being greatest in the group given the 15% algal protein diet and least in the control group. The weights of the testes were similar in all three groups, regardless of body weights. However, there was much less difference between the relative organ weights (g organ weight/100 g body weight) in the three groups (Table 2). The relative weights of the liver, kidneys,

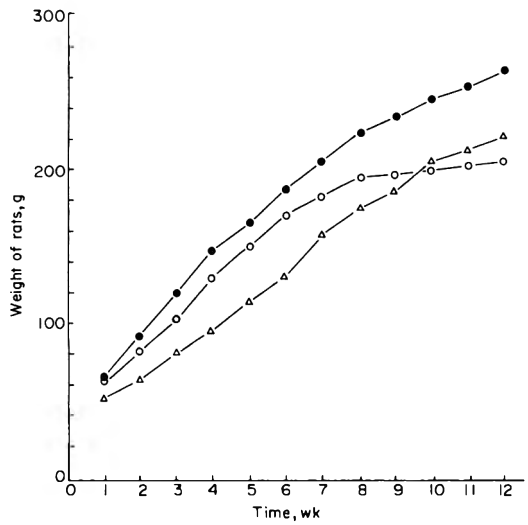

Fig. 2. Body weights of rats given diets containing 10% casein (—○—), 10% algal protein (—△—) or 15% algal protein (—●—) for 12 wk. Results are means for group of eight rats.

lungs, thyroid and testes were significantly greater in the control group.

The correlation between the weights of various organs was calculated as a correlation matrix using Hotelling's test (Morrison, 1967). A close and normal relationship was found between the weights of the liver, lungs, heart and kidneys in all three groups. No close relationship could be found between the weights of the brain, the testes and the adrenal, pituitary and thyroid glands, or between the weights of these organs and those of the liver, lungs, heart and kidneys. The weights of the organs in rats from the three experimental groups were compared by an overall multivariate one-way analysis of variance according to Scheffé (Morrison, 1967). At a confidence interval of 95% there was a difference between the groups in the weights of some of these organs, particularly in the weights of the adrenals and the testes.

Histopathological observations

Mild centrilobular fat infiltration was observed in the livers of rats fed the control diet but the livers of rats given the algal-protein diets were normal and no fat infiltration was observed. Neither cellular vacuolation nor distortion was found in the livers, hearts or kidneys of rats from any of the experimental groups.

Haematological data

There were no haematological abnormalities in any of the groups (Table 3). No significant differences were found between the groups in haemoglobin levels

Table 2. *Relative organ weights (g organ weight/100 g body weight) of rats fed diets containing 15 or 10% algal protein or 10% casein for 12 wk*

Organ	Relative organ weights of rats fed diets containing		
	10% casein	10% algal protein	15% algal protein
Liver	2·470 ± 0·167	2·270 ± 0·103**	2·240 ± 0·128
Kidneys	0·526 ± 0·026	0·456 ± 0·027**	0·488 ± 0·030†
Heart	0·266 ± 0·027	0·244 ± 0·017	0·260 ± 0·023
Lungs	0·650 ± 0·031	0·625 ± 0·027	0·600 ± 0·034*
Brain	0·709 ± 0·040	0·658 ± 0·039	0·667 ± 0·032
Adrenal glands	0·020 ± 0·00037	0·018 ± 0·00047	0·016 ± 0·00063
Pituitary gland	0·0017 ± 0·00024	0·0017 ± 0·00028	0·0016 ± 0·00039
Thyroid gland	0·0078 ± 0·0013	0·0065 ± 0·0011*	0·0065 ± 0·0011
Testes	0·947 ± 0·0050	0·883 ± 0·0592**	0·737 ± 0·0253**

Values are means ± SD for groups of eight rats. Those marked with asterisks differ significantly (Bartlett test) from the control value (*$P < 0·05$; **$P < 0·01$) and that marked with a dagger differs significantly from the value for the other group fed algal protein (†$P < 0·05$).

Table 3. *Haematological data for rats fed diets containing 10 or 15% algal protein or 10% casein for 12 wk*

Parameter	Values for rats fed diets containing		
	10% casein	10% algal protein	15% algal protein
Haemoglobin (g/100 ml whole blood)	15·4 ± 0·74	15·1 ± 0·79	14·6 ± 0·78
Red blood cells (10^6/mm^3)	6·1 ± 0·30	6·5 ± 0·47	6·2 ± 0·25
White blood cells (WBC)			
Total (10^3/mm^3)	8·6 ± 0·48	7·4 ± 0·30*	7·3 ± 0·27
Lymphocytes (% of total WBC)	75·3	74·6	73·1
Neutrophils (% of total WBC)	18·8	19·8	20·4
Eosinophils (% of total WBC)	2·1	2·3	2·6
Monocytes (% of total WBC)	3·8	3·3	3·1
Packed cell volume (%)	50·0 ± 1·4	48·0 ± 1·5	48·5 ± 1·4

Values are means or means ± SD for groups of eight rats and that marked with an asterisk differs significantly (Bartlett test) from the control value (*$P < 0·05$).

Table 4. *Activities of alanine aminotransferase and succinic dehydrogenase in the livers of rats fed diets containing 10 or 15% algal protein or 10% casein for 12 wk*

Enzyme	Enzyme activity* in livers of rats fed diets containing		
	10% casein	10% algal protein	15% algal protein
Alanine aminotransferase	93·45	103·75	101·20
Succinic dehydrogenase	0·58	0·59	0·49

*Alanine aminotransferase activity is expressed as mg pyruvate formed/g wet liver/10-min and succinic dehydrogenase activity is expressed as μg triphenyltetrazolium chloride reduced/10 min/mg wet liver.
Values are means of three independent estimations.

or in red blood cell counts. The total number of white blood cells was lower in blood from rats given the algal diets than in that from rats fed the control diet, although the differential white blood cell counts and the relative proportions of the various white cells were similar.

Statistical analysis of the data using Hotelling's test (Morrison, 1967) revealed a close and normal relationship between the haemoglobin content, red and white blood cell counts and packed cell volume within each dietary group.

Urine analyses

No sugar, bile salts, pigment or ketone bodies were detected in the urine of rats from any of the dietary groups. The urine was alkaline and small quantities of urinary albumin were detected in all groups. Microscopic examination did not show the presence of any cells in the urine or provide evidence of any infection. However, some calcium oxalate and triphosphate crystals were observed in the urine of rats from all the groups.

Cholesterol levels

The total liver cholesterol level was significantly ($P < 0·01$) lower in rats fed the algal diets than in the control animals, the mean values of eight observations being 2·14, 1·75 and 1·63 mg cholesterol/g liver in the control, 10 and 15% algal-protein dietary groups, respectively. No deposition of cholesterol crystals was observed in the livers of rats in any group. Serum cholesterol levels were similar in all three groups.

Liver biochemistry

There were no significant differences in hepatic lipid and protein levels between the three groups. Alanine aminotransferase activity was slightly higher in rats given algal diets than in the controls, and there was a small reduction in the activity of succinic dehydrogenase in rats fed the 15% algal-protein diet. However the activities of both of these enzymes were still within the normal limits for rats fed the control diet (Table 4).

Gel electrophoretic pattern of serum proteins

There were no qualitative or quantitative differences between the serum proteins of the control group and those of the groups given the algal diets. The proteins separated into at least ten different components, which were broadly classified into albumins (having the highest electrophoretic mobility) and globulins. The latter were further classified into α_1, α_2, β and γ globulins on the basis of their mobility and distribution in the gel. Study of the gel electrophoretic and microdensitometric scanning patterns showed that the number of protein components, their electrophoretic mobility and their relative percentage distribution were similar in all three groups.

DISCUSSION

The feeding of drum-dried alga to albino rats at dietary protein levels of 10 and 15% for 12 wk did not have any significant toxicological or histopathological effects. Algal proteins used as a sole protein source have been reported to be comparable with casein in their ability to support growth (Soeder & Pabst, 1975; Venkataraman, Becker, Khanum & Mathew, 1977). We have discussed the nutritional quality of algal proteins in previous papers (Becker *et al.* 1976a,b; Subbulakshmi, Becker & Venkataraman, 1976; Venkataraman, Becker & Khanum, 1977). Although in the present study the algal proteins were fed for only 3 months, the data obtained from urine analyses and from haematological, toxicological and histopathological tests have indicated that this new source of protein is harmless.

The relative organ weights of animals in the two experimental groups were similar to or relatively less than those of the animals in the control group. The relative weights of the different organs within one animal were also similar in all the three groups, with obtained did not indicate any pathological alterations in any of the three groups, despite a minor decrease in the white blood cell count in the rats given the algal-protein diets. Alterations in hepatic enzyme levels can none of the organs showing an abnormal increase or decrease in weight in response to treatment; the testes were approximately the same size in all rats regardless of their body weight. The haematological data be interpreted as a metabolic regulatory effect and so changes in the activities of enzymes such as succinic dehydrogenase and alanine aminotransferase may reflect dietary deficiencies or toxicity. The activities of these enzymes were unaffected by feeding algal proteins.

Total liver cholesterol levels were lower in rats fed the algal-protein diet than in the casein-fed control group. This hypocholesterolaemic effect indicates an

added advantage of algal-protein diets, since a high dietary level of cholesterol is one of the factors that has been linked to the incidence of coronary heart disease and arteriosclerotic lesions (Mathur, Gupta & Sharma, 1965). It has been established that consumption of legumes and some other dietary constituents lowers serum and liver cholesterol levels (Kowale & Misra, 1975) and hypocholesterolaemic effects have been observed with another unicellular alga, Chlorella, in Japan (Okuda, Hasegawa, Sonoda, Okabe & Tanaka, 1975). The cholesterol-lowering effects of the algal proteins used in the present study will be evaluated more thoroughly.

Algae are intended for use only as a supplementary and not as the sole protein source. The data available so far do not indicate any toxicological effects as a result of including algae, grown in clean water, in food or feed.

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EFFECT OF BETEL-QUID CHEWING ON NITRITE LEVELS IN SALIVA

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Abstract—A study was made of the effects of chewing betel quid, alone or with tobacco, on salivary levels of nitrite and other factors of importance in the formation of nitrosamines, and the possible relevance of the findings to the development of oral cancer was assessed. When a betel quid was chewed after ingestion of a lunch that was not rich in nitrates, saliva samples taken 1 hr later showed an increase in nitrite level, except in habitual chewers of tobacco. Subsequent studies confirmed that a substantial rise in salivary nitrite followed betel-quid chewing by volunteers who never chewed tobacco, but no such increase occurred when tobacco and betel quid were chewed together. Basal levels of salivary nitrite were also lower in habitual tobacco chewers than in non-chewers. Free amino-nitrogen levels in the saliva were not affected significantly either by habitual tobacco chewing or by the experimental chewing of betel quid or betel with tobacco. However, salivary pH was increased by both types of test chew, and particularly by the betel quid alone, while thiocyanate levels were significantly higher in habitual tobacco chewers than in non-chewers and were further increased after the chewing of betel quid with tobacco.

INTRODUCTION

It is well known that human saliva contains detectable quantities of nitrite (Savostianov, 1937; Varady & Szanto, 1940) and also that nitrates released from the salivary ducts are reduced to nitrites by the action of nitrate reductase secreted by bacteria in the oral cavity (Goas & Biswell, 1961). Thus nitrites in saliva are always in a dynamic state, being formed by micro-organisms and at the same time consumed by chemical reactions and other micro-organisms. Tannenbaum, Sinskey, Weisman & Bishop (1974) have identified various nitrate-reducing strains of bacteria present in the oral cavity. The concentration of nitrites in saliva is regulated by such factors as the type of food and the flora of oral cavity. Nitrate levels in vegetables, meats and dairy products and nitrite levels in saliva have been investigated recently by Hickey & Clelland (1978), Klein, Gaconnet, Poullain & Debry (1978) and Sieber & Blanc (1978).

It has been clearly shown that nitrites combine with amines at acidic or almost neutral pH *in vivo* as well as *in vitro* (Sen, Smith, Schwinghamer & Marleau, 1969; Tannenbaum, Archer, Wishnok & Bishop, 1978; Telling, Hoar, Caswell & Collings, 1976; Walters, Dyke, Saxby & Walker, 1976). Since the rate of formation of nitrosamines follows the general equation in which: rate = k[amine] [nitrate]n, where $n = 2$ or more (Sander, Schweinsberg, Labar, Burkle & Schweinsberg, 1975), it is possible that an increase in nitrite levels may promote the biosynthesis of nitrosamines.

In India, oral cancer is one of the most common types of cancer and epidemiological data collected so far have indicated a causal relationship between oral cancer and chewing habits (Paymaster, 1962; Pindborg, Barones & Roed-Peterson, 1968). It was observed by Notani & Sanghvi (1976) that people with vegetarian habits were less protected from oral cancer than those who supplemented their diet with egg, fish or meat proteins, an important observation in view of the fact that the diets of Indian people contain a high proportion of nitrate-rich vegetables, beans and pulses. Therefore an initial study of nitrite levels in the saliva of a small randomly selected group was undertaken among scientific and technical staff working at the Cancer Research Institute, Bombay, and the variation of these nitrite levels following the chewing of betel quid with or without tobacco was studied. Salient observations on these studies are presented in this paper.

EXPERIMENTAL

Test personnel. The volunteers were members of the scientific and technical staff of the Cancer Research Institute, Bombay. They were 20–25 yr old and of either sex and included both vegetarians and non-vegetarians. However, it must be borne in mind that even the non-vegetarian people in India subsist to a great extent on vegetables, beans and pulses, and consume meat or fish preparations only once or twice a week.

Experimental procedures

Collection of saliva samples. The donors were requested to rinse their mouths with water, and then to expectorate during the next 15–20 min into wide-mouthed plastics bottles until a 5-ml sample had been collected. The bottles had screw caps and contained 0·1 ml 0·1 N-NaOH to inhibit any bacterial growth. No attempt was made to separate any exfoliated cells either by centrifugation or filtration, since the aim was to test the nitrite levels in the naturally occurring milieu of the oral cavity.

Studies of normal saliva. In initial experiments, saliva samples were collected from groups of 12–18 volunteers at 06.00 hr (fasting sample), at 10.00 hr following a breakfast consisting of cereals, such as rice

or bread, and tea or coffee, and at 14.00 hr after a lunch consisting of rice, vegetables, pulses and wheat pancakes or chapati. In subsequent experiments, the samples were collected at 10.00 hr. One group of six volunteers was used to follow the changes in nitrite levels in 10.00-hr samples over a 10-day period.

Effects of betel-quid chewing on nitrite levels. The betel quid consisted of betel leaf, betel nut, trace amounts of lime and catechu, and all the quid was purchased from the same shop at one time. A betel quid taken without tobacco is generally chewed within 15–20 min and the chew is gradually ingested; chewing the quid with tobacco stimulates the secretion of saliva, which is spat out, so most of the chew is also spat out within 15–20 min, although some of the secretions are undoubtedly swallowed during the chewing process. Following the collection of a sample of saliva from each of seven volunteers 1 hr after they had eaten a lunch containing no nitrate-rich pulses or vegetables, each donor was given a betel quid, and three further saliva samples were collected at intervals of 1 hr after ingestion of the complete chew.

Effects of betel/tobacco chewing on saliva. Larger test groups (9–22 test subjects) were used to study the effect of the simultaneous chewing of tobacco and betel on salivary-nitrite levels. Following collection of a saliva sample at 10.00 hr, the habitual chewers of tobacco were given a betel quid together with 2–3 g dried crumpled tobacco leaves, while the other volunteers chewed only a betel quid. A further saliva sample was taken from each test subject 1 hr after the chewing process had been completed. Some saliva samples were taken 1 hr after the chewing of a betel quid or of the quid with tobacco for determinations of pH, thiocyanate content and free amino nitrogen and the findings were compared with those for the relevant 10.00-hr (pre-chewing) sample.

Analytical methods. The nitrite content of saliva was measured by a modified colorimetric method based on a recommended standard technique (International Standards Organization, ISO 3091–1975). Thiocyanate was measured colorimetrically by the method of Johnson (1916), and pH was measured immediately after sample collection (in bottles containing no alkali) on a Beckman pH meter. Free amino nitrogen was measured colorimetrically using the ion-exchange method of Miller & Rice (1963).

RESULTS

Mean variations of salivary nitrite levels with time are shown in Table 1 for groups studied in three different experiments. Some of the donors from Experiment 1 were also included in the second or third experiment. The tabulated data show that the post-prandial saliva samples, collected at 14.00 hr, had the highest nitrite content, probably as a result of the recent ingestion of pulses and vegetables rich in nitrates.

In the subsequent study of salivary samples taken from six individuals at 10.00 hr on 4 days out of a total of 10, no attempt was made to maintain uniformity in meals and, as the donors were randomly selected, there was considerable variation in the salivary nitrite content of the different individuals at any given time. Table 2 indicates, however, that the individual nitrite values varied two- to threefold under normal conditions, a variation probably due to the variation in diet on the different days.

Table 3 demonstrates that in the study of the effects of betel-quid chewing on salivary nitrite, all the sample donors except two showed an increase in salivary nitrite 1 hr after the chewing period, but the

Table 1. *Variation of nitrite levels with time in the saliva of normal individuals*

Experiment no.	Mean levels of nitrite (ppm) in samples taken at		
	06.00 hr	10.00 hr	14.00 hr
1	21·3 ± 1·9 (12)	12·5 ± 1·0* (13)	36·1 ± 4·9† (11)
2	29·4 ± 2·9 (16)	17·0 ± 1·4* (16)	60·7 ± 8·1† (16)
3	26·0 ± 3·2 (18)	15·5 ± 1·0 (18)	40·4 ± 8·7 (15)

Values are expressed as the mean ± SEM for the number of samples indicated in brackets. Those marked with superscripts differ significantly ($P < 0·05$ by Student's t test) either from the 06.00-hr value (*) or from the 10.00-hr value (†).

Table 2. *Nitrite levels in saliva samples taken from the same individuals at 10.00 hr on different days*

Donor	Levels of nitrite (ppm) in saliva collected on day			
	1	3	5	10
S.V.B.	6·1	20·0	10·3	13·2
N.M.S.	5·0	12·5	13·1	18·6
G.B.M.	13·3	13·0	26·4	18·4
A.V.D.	25·9	45·3	—	10·2
H.S.H.	13·5	13·0	25·2	10·9
A.V.W.	8·2	9·0	13·1	15·0

Table 3. *Nitrite levels in saliva samples collected at intervals after betel-quid chewing*

Donor	Levels of nitrite (ppm) in samples taken at			
	0 hr*	1 hr†	2 hr†	3 hr†
S.V.B.	15·1	19·9	20·3	16·0
N.A.S.	5·4	20·3	8·1	5·6
G.B.M.	14·0	20·7	—	18·5
D.S.	4·2	13·5	32·7	7·1
S.N.T.	35·2	81·0	19·7	—
H.V.B.§	9·2	7·0	9·1	9·1
T.A.W.§	14·5	13·7	7·0	7·5

*Sample taken immediately before chewing period.
†Time after end of chewing period.
§Habitual chewer of tobacco.

levels fell again within another 1–2 hr. It may be pertinent to mention here that the two exceptions were habitual tobacco chewers.

The experiment on the effect of chewing betel quid with tobacco was repeated several times, in two cases in conjunction with parallel studies in volunteers chewing the betel quid alone (Table 4). Test subjects with salivary nitrite levels in the lower range of their respective groups were selected for these experiments. The results clearly indicate that betel quid chewed alone increased the nitrite levels substantially. This is understandable since betel leaf is rich in nitrates. However, a similar increase was not observed in donors who chewed betel quid with tobacco. Furthermore, the salivary-nitrite levels of habitual tobacco chewers, as determined prior to the test chewing, were generally lower than the normal levels in non-chewers.

The mean values for pH, free amino-nitrogen and thiocyanate in samples taken before chewing began and 1 hr after the chewing of betel quid with or without tobacco are shown in Table 5. This shows an increase in the pH of saliva after either type of chewing, the increase in the group chewing betel quid without tobacco being statistically significant. The considerable amount of free amino nitrogen present in the saliva of both habitual chewers and non-chewers was not affected significantly by the experimental chewing. Prior to chewing, the saliva of both chewers and non-chewers contained a significant quantity of thiocyanate but the level was significantly higher ($P < 0{\cdot}05$) in the habitual tobacco chewers than in non-chewers. The thiocyanate levels in the saliva of tobacco chewers had increased further after the experimental chewing, but this increase was not statistically significant.

DISCUSSION

The volunteers randomly selected for this investigation represented a broad spectrum of the castes and communities located in the western region of India. While not under-nourished, they subsist essentially on a low-protein and mainly vegetarian diet. It is clear from the data presented that the nitrite levels in the saliva of those who are not tobacco chewers are higher than those reported in the literature for western populations (Tannenbaum *et al.* 1974). In fact, these values compare closely with those reported for

Table 4. *Nitrite levels in saliva samples collected before and after the chewing of betel quid with or without tobacco*

Test group†	Experiment no.	Mean nitrite levels (ppm) in samples taken	
		Before chewing‡	After chewing‡
Betel alone	1	11·3 ± 2·1 (22)	28·7 ± 5·0* (22)
	2	9·5 ± 1·1 (19)	21·6 ± 4·1* (19)
Betel + tobacco	1	5·5 ± 0·5 (11)	4·3 ± 0·7 (11)
	2	6·2 ± 0·5 (14)	8·9 ± 0·9 (14)
	3	5·3 ± 1·0 (11)	9·2 ± 2·6 (11)
	4	8·4 ± 2·1 (9)	11·0 ± 2·6 (9)

†Betel quid with tobacco was given to the habitual chewers of tobacco and betel quid alone to non-chewers of tobacco.
‡Samples were taken at 10.00 hr, immediately before chewing commenced, and 1 hr after the chewing was completed.
Values are expressed as the mean ± SEM for the number of samples indicated in brackets. Those marked with an asterisk differ significantly ($P < 0{\cdot}05$ by Student's t test) from the corresponding pre-chewing value.

Table 5. *Salivary pH and content of free amino-nitrogen and thiocyanate before and after the chewing of betel quid with or without tobacco*

Test group‡	Sampling time§	pH	Free amino-nitrogen (μg/ml saliva)	Thiocyanate (mmol/ml saliva)
Betel alone	Before	6·9 ± 0·1 (8)	692 ± 86 (10)	0·20 ± 0·04† (10)
	After	7·5 ± 0·17* (8)	484 ± 101 (10)	0·18 ± 0·01† (10)
Betel + tobacco	Before	6·5 ± 0·28 (6)	643 ± 77 (5)	0·35 ± 0·05 (5)
	After	7·1 ± 0·1 (6)	783 ± 104 (5)	0·47 ± 0·07 (5)

‡Betel quid with tobacco was given to the habitual chewers of tobacco, and betel quid alone to non-chewers of tobacco.

§Samples were taken immediately before the chewing of the test quid and 1 hr after completion of the chew.

Values are expressed as the mean ± SEM for the number of samples indicated in brackets. Those marked with a superscript differ significantly ($P < 0·05$ by Student's t test) either from the corresponding value before chewing in the same group (*) or from the corresponding value in the tobacco-chewing group (†).

the saliva of people from villages in the Caspian Littoral that are reported to show a high incidence of oesophageal cancer (Castegnaro, Walker, Preussmann, Eisenbrand & Spiegelhalder, 1978). It is noteworthy that nitrite levels in post-prandial saliva samples are also high, a clear indication of the influence of diet on these levels. Tannenbaum *et al.* (1974) did not observe a similar increase after lunch, but the subjects in his studies did not have a meal that was rich in nitrates. On the other hand, there is ample evidence of an increase in salivary nitrites after the drinking of beetroot juice or other vegetable juices rich in nitrates (Spiegelhalder, Eisenbrand & Preussmann, 1976; Tannenbaum, Weisman & Fett, 1976). The high nitrite levels in the saliva of non-chewers could provide a partial explanation for the lack of protection from oral cancer in vegetarian communities without tobacco-chewing or smoking habits (Notani & Sanghvi, 1976). It is possible that these high nitrite levels are one of the factors in an apparent predisposition of vegetarians to oral cancer.

It is rather puzzling that the habitual tobacco chewers did not show nitrite values as high as those in non-chewers. They also failed to show any increase in salivary nitrites after chewing betel quid with or without tobacco. The latter finding indicates that the tobacco does not inhibit the increase in nitrite content directly. It is possible that the oral mucosa and salivary ducts of these habitual tobacco chewers are not functioning optimally, because the mucosa has been damaged by the continual chewing of tobacco, and thus the release of nitrates from the salivary ducts is hampered.

The measurements of salivary pH, of the levels of thiocyanate ions, which catalyse the nitrosation of amines (Boyland, Nice & Williams, 1971) and of free amino nitrogen, a measure of free amino acids liable to form amines by decarboxylation, were of interest as indications of the extent to which the salivary milieu might encourage the formation of nitrosamines in the oral cavity. The levels of free amino nitrogen demonstrated and the presence of thiocyanate supported the possibility of such *in vivo* nitrosamine formation. In view of the finding that thiocyanate levels were higher in habitual tobacco chewers than in non-chewers, it is interesting to note that Schievelbein, Werle, Schulz & Baumeister (1969) observed higher thiocyanate levels in the saliva of tobacco smokers than of non-smokers. Chewing betel quid without tobacco once or twice a day is a common habit among men and women in India and this practice may further enhance the period of exposure of the oral mucosa to increased nitrites and possibly to nitrosamines.

However, these observations were made on a limited number of samples and before any firm conclusions can be drawn it will be essential to carry out a large and comprehensive survey of salivary nitrite in chewers and non-chewers and to test the effect of eating nitrate-rich vegetables and pulses on salivary nitrites in tobacco chewers. Other essential studies will involve identification of the bacterial flora in the oral cavity of the Indian population and correlation of the status of oral hygiene with salivary nitrites. It will also be of interest to establish whether nitrosamines can be detected in the chew of betel quid as well as in salivary secretions subsequent to the chewing of tobacco. Some work along these lines is already in progress.

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CHEMISTRY OF NITROSO-COMPOUNDS. PART 16*. FORMATION OF *N*-NITROSAMINES FROM DISSOLVED NOCl IN THE PRESENCE OF ALKANOLAMINES AND RELATED COMPOUNDS

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Abstract—The formation of carcinogenic *N*-nitrosamines (e.g. *N*-nitrosopiperidine) from dissolved NOCl gas in the presence of eight alkanolamines (triethanolamine, diethanolamine, *N*-methylethanolamine, *N*,*N*-diethylethanolamine, *N*-acetylethanolamine, *N*-nitrosodiethanolamine, *N*-methyl-*N*-nitrosoethanolamine and choline chloride) and secondary amines in aqueous solution (usually 0·1 M-NaOH) at 25°C is reported. In the absence of alkanolamines and with an approximately sixfold excess of NOCl, about 35% of the amine is converted into *N*-nitrosamine in less than 3 min. In the presence of alkanolamines, the reactions are slower but often much more extensive. With 0·05 M-*N*-nitrosodiethanolamine (the best promotor examined) about 85% of 2×10^{-3} M-piperidine in 0·1 M-NaOH at 25°C is converted to *N*-nitrosopiperidine in 38 min by a sixfold excess of NOCl. These reactions are considered to proceed *via* an alkyl nitrite intermediate generated from the alkanolamine and the NOCl, which then nitrosates the secondary amine in a rate-limiting step. The most reactive intermediates derive from alkanolamines bearing strong electron-withdrawing substituents. Similar reactions are observed with N_2O_4 in place of NOCl, and it is suggested that in this way, NO_x pollutants may contribute to the formation of *N*-nitrosamines in consumer products containing alkanolamines as emulsifiers.

INTRODUCTION

N-Nitrosodiethanolamine, a known animal carcinogen (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Hilfrich, Schmeltz & Hoffmann, 1978), has been detected recently in some cosmetics and shampoos (Fan, Goff, Song, Fine, Arsenault & Biemann, 1977) and cutting fluids (Fan, Morrison, Rounbehler, Ross, Fine, Miles & Sen, 1977; Rappe & Zingmark, 1978) containing ethanolamines. The origin of this contaminant is not completely understood.

One plausible explanation is that diethanolamine reacts directly with a nitrosating agent such as adventitious nitrogen oxides (Challis & Kyrtopoulos, 1978 & 1979) or, in the case of cosmetics, with aliphatic nitro compounds (Fan, Vita & Fine, 1978; Schmeltz & Wenger, 1979) present as bacteriocides. Alternatively, triethanolamine itself may interact with these nitrosating agents to form a nitrosonium salt, $[(HOCH_2CH_2)_3NN = O]^+X^-$, which then undergoes dealkylation similar to that observed in the nitrosation of other tertiary amines (Lijinsky, Keefer, Conrad & Van de Bogart, 1972; Smith & Loeppky, 1967). The dealkylation of the nitrosonium salt, however, is likely to be very much slower than the direct *N*-nitrosation of diethanolamine.

Very recently it has been shown (Challis & Shuker, 1979a) that alcohols bearing β-electron-withdrawing substituents (e.g. ethylene glycol) catalyse the formation of many *N*-nitrosamines from nitrosyl gases and secondary amines (other than diethanolamine) under exceptionally mild conditions. We have examined these reactions further and found similar effects by several alkanolamines. In fact, one of the most powerful promoters is *N*-nitrosodiethanolamine itself.

EXPERIMENTAL

Reagents, substrates and products. NOCl (99%; Matheson, East Rutherford, NJ) and N_2O_4 (99·5%; Matheson) were used without further purification. Piperidine, morpholine and *N*-methylpiperazine were dried over solid KOH and then distilled. Choline chloride (Sigma Chemical Co., Poole, Dorset) was then used as supplied.

Diethanolamine, triethanolamine and *N*-methylethanolamine were all vacuum-distilled and the middle cuts were taken. *N*-Acetylethanolamine was prepared by acetylation of ethanolamine using acetic anhydride followed by vacuum distillation (Heyns & Bebenburg, 1955): b.p. 124–126°C/0·8 Torr, n_D^{25} 1·4702, lit. (Heyns & Bebenburg, 1955) b.p. 177–179·5°C/18·5 Torr, n_D^{23} 1·4715. *N*,*N*-Diethylethanolamine was obtained from the reaction of diethylamine with 2-chloroethanol (Horne & Shriner, 1932); the crude product was purified by distillation to give a colourless liquid: b.p. 60°C/18 Torr, $n_D^{22.5}$ 1·4417; lit. (Horne & Shriner, 1932) b.p. 56–57°C/15 Torr, n_D^{25} 1·4400.

N-Methyl-*N*-nitrosoethanolamine was synthesized by treating *N*-methylethanolamine with acidified nitrite (Ogimachi & Kruse, 1961) and the product was purified by vacuum distillation: b.p. 90–92°C/0·3 Torr,

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n_D^{22} 1·4782; lit (Ogimachi & Kruse, 1961) b.p. 110·5–111·5°C/Torr, n_D^{25} 1·4778. *N*-Nitrosodiethanolamine was prepared by the nitrosation of diethanolamine following the procedure of Jones & Wilson (1949); vacuum distillation of the product resulted in excessive decomposition, so it was purified by preparative HPLC (Waters Associates Prep LC/System 500) using ethyl acetate as the eluent. Evaporation of the solvent gave a pale yellow oil n_D^{22} 1·4985, ν_{max} 3360, 1440 cm^{-1}; lit. (Jones & Wilson, 1949) n_D^{20} 1·4849; found, C 35·59, H 7·81, N 20·54%—$C_4H_{10}N_2O_3$ requires C 35·82, H 7·51, N 20·88%. Authentic *N*-nitrosopiperidine, *N*-nitrosomorpholine and *N*-methyl-*N*-nitrosopiperazine were all prepared by nitrosation of the relevant amines with acidified nitrite (Vogel, 1956).

Reaction methods. For reactions in the absence of alkanolamines, the aqueous reaction solution (5 ml) containing either 0·1 M-NaOH or phosphate buffer was placed in a 50-ml conical flask and then purged with oxygen-free nitrogen for *c.* 30 min. The neat amine was then added to this solution by means of a microlitre syringe. The flask was closed with a Subaseal stopper and brought to 25°C by immersion in a water bath. With a syringe 0·5–10-ml quantities of the nitrosyl gas (measured at 1 atm and 25°C) were injected into the flask through the Subaseal stopper. After the flask had been shaken for *c.* 2–3 min, small aliquots of the aqueous solution were withdrawn for immediate analysis. Independent tests established that the amounts of reaction products did not change with time over about 2 hr and that the reaction was complete in much less than the usual 2–3-min shaking period.

A slightly modified procedure was used for reactions in the presence of an alkanolamine or related catalyst. After the reaction solution had been purged with oxygen-free nitrogen, the requisite amount of catalyst was added before the reaction flask was closed and thermally equilibrated as described. The nitrosyl gas was then added and the flask was shaken for 2 min, after which the neat amine was added *via* the Subaseal stopper using a microlitre syringe and was thoroughly mixed with the aqueous solution. At timed intervals, small aliquots of the reaction solution were withdrawn similarly for immediate analysis. These reactions were followed for much longer periods (up to 24 hr) until successive aliquots showed a constant amount of *N*-nitrosamine. Except for very rapid reactions in the presence of *N*-nitrosodiethanolamine and choline chloride catalysts, aliquots taken within 2–3 min of the addition of the amine showed negligible levels of *N*-nitrosamine product. This confirms that the nitrosyl gas reacts completely with the catalyst and solvent before the amine is added.

The dead volume above the reaction solutions in all experiments was *c.* 60 ml. Thus, the partial pressure of nitrosyl gas in the reaction flask prior to mixing varied from 0·167 to 0·008 atm for NOCl and from 0·167 to 0·1 atm for N_2O_4.

Analytical procedures. *N*-Nitrosopiperidine, *N*-nitrosomorpholine and *N*-methyl-*N*-nitrosopiperazine were assayed by GLC against standard solutions of authentic materials prepared in the same solvent as that used for the compound under investigation. Typically, 0·5–1 μl of the reaction solution was injected directly onto the column (80% Carbowax 20 M plus 2% KOH on Chromosorb W, 80–100 mesh at 190°C) of a Perkin Elmer F33 Gas Chromatograph with a P–N-specific FID using N_2 carrier gas. Concentrations were linearly related to peak heights, which were reproducible for any given sample to better than ±3%.

The concentration of NO_2^- formed by concurrent hydrolysis was determined by the modified Shinn's procedure (Kershaw & Chamberlin, 1942). Independent checks established that decomposition of the *N*-nitrosamine products (to release HNO_2) was insignificant during the NO_2^- assay (<0·5%).

RESULTS

Basic reactions

Essentially, the experiments involved manual mixing of about 60 ml of a dilute mixture of the nitrosyl gas (NOX, where X = Cl^- or NO_3^-) in nitrogen with 5 ml of either aqueous amine or alkanolamine solution at 25°C. For the latter, an appropriate secondary amine was then added after all the nitrosyl gas had reacted with either the alkanolamine or the solvent. The solution also contained either 0·1 M-NaOH or 0·5 M-phosphate buffer (pH 6·85) to prevent any reaction by the usual acid-catalysed nitrosation pathways following hydrolysis of the nitrosyl gas and formation of an alkyl nitrite. In the absence of alkanolamine, both the hydrolysis (equation 1) and *N*-nitrosation (equation 2) reactions appeared to be complete within a few seconds of the mixing of the gaseous and aqueous phases.

$$NOX + 2HO^- \rightleftharpoons NO_2^- + X^- + H_2O \quad (1)$$

$$R_2NH + NOX \xrightarrow{HO^-} R_2NNO + X^- + H_2O \quad (2)$$

In the presence of alkanolamine, formation of the corresponding alkyl nitrite (equation 3) appeared to proceed equally rapidly.

$$NOX + HOCH_2CH_2NR_2 \xrightarrow{HO^-} R_2NCH_2CH_2ONO + X^- + H_2O \quad (3)$$

We believe that reaction of the nitrosyl gas with either amine or alkanolamine occurs principally in the aqueous phase following absorption of the gas. Evidence to support this conclusion is the low volatility of alkanolamines and the inhibition of *N*-nitrosamine formation when 0·5 M-NaN_3 is added to the reaction solution. NaN_3 is known (Stedman, 1960) to convert nitrosating agents rapidly to a mixture of N_2 and N_2O.

To simplify the mechanistic interpretation of the results, most experiments were carried out with gaseous NOCl rather than N_2O_4. Previous experiments had shown that NOCl acts only as a nitrosating agent (Challis & Shuker, 1979b), whereas N_2O_4 produces a mixture of *N*-nitrosamines and *N*-nitroamine (Challis & Kyrtopoulos, 1978 & 1979). NOCl is only slightly dissociated (equation 4) under the reaction conditions, as extrapolation of measurements at 100–200°C (Beeson & Yost, 1939) gives

$Kp = 7{\cdot}4 \times 10^{-8}$ atm for equation 4 at 25°C.

$$2NOCl \rightleftharpoons 2NO + Cl_2 \quad (4)$$

Thus at the low partial pressures in our experiments ($P_{NOCl} = 0{\cdot}167$–$0{\cdot}001$ atm), the dissociation of NOCl is probably less than 1% in the gaseous phase and even lower in the aqueous solution (Shaw & Vosper, 1971). N_2O_4, however, is more extensively dissociated (equation 5) under similar conditions ($Kp = 0{\cdot}15$ atm at 25°C; Gratzel, Henglein, Lilie & Beck, 1969), so that nitration as well as nitrosation products are formed.

$$N_2O_4 \rightleftharpoons 2NO_2 \quad (5)$$

The actual concentrations of nitrosyl gas added could not be estimated very accurately from the volume of gas injected, particularly when using N_2O_4 because of its dissociation. These concentrations were therefore deduced from the yield of NO_2^- plus the amount of *N*-nitrosamine, a sum referred to below as the 'titrable nitrite concentration' (TNC). The yield of *N*-nitroamine is not taken into account because this reaction (equation 6) concurrently produces 1 mol NO_2^-.

$$R_2NH + N_2O_4 \xrightarrow{HO^-} R_2NNO_2 + NO_2^- + H_2O \quad (6)$$

Hydrolysis of 1 mol NOCl produces 1 mol NO_2^- (equation 1). The concentration of NOCl is therefore equal to the TNC. For N_2O_4, Saltzmann (1954) found that 1 mol NO_2^- is produced/mol N_2O_4 from hydrolysis at $P_{N_2O_4} > 0{\cdot}005$ atm.

[NO_2^- may result from hydrolysis of either N_2O_4 or NO_2 (equations 7 and 8).

$$N_2O_4 \xrightleftharpoons{2HO^-} NO_2^- + NO_3^- + 2H_2O \quad (7)$$

$$\downarrow\uparrow$$

$$2NO_2 \xrightleftharpoons{2HO^-} 2NO_2^- + 2HO^{\cdot} \quad (8)$$

It is the latter reaction that results in the formation of > 1 mol NO_2^-/mol N_2O_4. This occurs only for highly dissociated N_2O_4 at low $P_{N_2O_4}$.] Our reactions were carried out at significantly higher $P_{N_2O_4}$ but this relationship was checked by showing that equal amounts of NO_2^- and NO_3^- were obtained from the hydrolysis of N_2O_4 in 0·1 M–NaOH in the absence of amine or alkanolamine. Thus the concentration of N_2O_4 is equal to the TNC.

Reactions of NOCl with piperidine in the absence of alkanolamines

Yields of *N*-nitrosopiperidine obtained in 0·1 M-aqueous NaOH at 25°C from different initial concentrations of NOCl and piperidine are summarized in Table 1. These results refer to samples analysed 3 min after the addition of the NOCl, but the reactions were complete within a few seconds and the yields remained unchanged over a further period of 2 hr. Analysis of these data show that the formation of *N*-nitrosopiperidine follows equation 9 (Challis & Shuker, 1979b).

$$\text{Rate} = k_2[NOCl][\text{piperidine}] \quad (9)$$

A similar equation applies to the reaction with N_2O_4 (Challis & Kyrtopoulos, 1978). With 2×10^{-3} M-piperidine *c.* 6·1% NOCl and 3·6% N_2O_4 reacts to form *N*-nitrosopiperidine. The remainder is hydrolysed to give NO_2^- and Cl^- from NOCl, and NO_2^- and NO_3^- from N_2O_4. With excess piperidine, however, all of the NOCl (Challis & Shuker, 1979b) and about 50% of the N_2O_4 (Challis & Kyrtopoulos, 1978) is converted to *N*-nitrosopiperidine.

Reactions of nitrosyl gases with amines in the presence of alkanolamines

As described under Experimental, these reactions were carried out by first reacting the nitrosyl gas with aqueous alkanolamine and then adding the relevant amine after *c.* 2 min.

The reaction of piperidine with NOCl plus triethanolamine was examined in most detail. Results summarized in Table 2 show that, in 0·1 M-NaOH at 25°C, both the final yield of *N*-nitrosopiperidine and its initial rate of formation are dependent on the initial concentrations of piperidine, NOCl and triethanolamine. Thus, the reaction rate probably follows equation 10:

$$\text{Rate} = k_3[\text{Piperidine}][NOCl][(HOCH_2CH_2)_3N] \quad (10)$$

No product formed in phosphate buffer at pH 6·85, however, even with the highest concentrations of reac-

Table 1. *Variation in yield of* N-*nitrosopiperidine with initial concentrations of NOCl and piperidine in 0·1* M-*NaOH at 25°C*

10^3[TNC] (M)	10^3[Piperidine] (M)	10^3[*N*-Nitrosopiperidine]* (M)
9·22	2·02	0·62
16·8	2·02	1·03
21·3	2·02	1·27
28·5	2·02	1·70
30·0	2·02	1·69
30·0	4·06	3·15
30·0	8·12	5·35
30·0	20·3	9·60
30·0	60·9	20·0
30·0	203·0	21·8
30·0	505·0	26·2
30·0	812·0	31·0
30·0	1200·0	35·1

*After 3 min.

Table 2. *Effect of reactant concentrations on the initial rate and limiting yield for reaction of NOCl plus triethanolamine with piperidine in 0·1 M-NaOH at 25°C*

10^3[TNC]* (M)	10^3[Piperidine] (M)	10^3[$(HOCH_2CH_2)_3N$] (M)	10^7 Initial rate† (mol litre^{-1}sec^{-1})	10^4[*N*-Nitrosopiperidine]‡ (M)
11·4	2·03	50	6·83	12·1
11·4	1·02	50	3·54	6·6
11·4	0·51	50	1·75	3·5
11·4	2·03	25	3·58	6·4
11·4	2·03	10	2·04	4·15
6·0	2·03	50	2·08	4·6
3·0	2·03	50	1·33	2·55

*Equal to initial [NOCl] (see text).
†From reaction over initial 6 min.
‡After 24 hr.

tants. This established that only the unprotonated amine was reactive.

The combination of NOCl with triethanolamine was also used to examine the influence of amine reactivity (basicity). Comparative results for piperidine (pK_A 11·12) and morpholine (8·33) in 0·1 M-NaOH and for *N*-methylpiperazinium ion (5·11) in phosphate buffer at pH 6·85 are shown in Fig. 1. The highest yield and fastest rate applies to piperidine, which shows that the nitrosating agent derived from NOCl and triethanolamine is more selective than NOCl itself (Challis & Shuker, 1979b). On this basis, the higher reactivity of *N*-methylpiperazinium ion compared to morpholine (Fig. 1) is unexpected, but is related to the different reaction conditions. At pH 6·85 (used for *N*-methylpiperazinium ion), the nitrite ester derived from triethanolamine (pK_A 7·8) and NOCl should be extensively protonated. This reagent ($ONOCH_2CH_2^+NH(CH_2CH_2OH)_2$) should be more reactive than the neutral ester ($ONOCH_2CH_2N(CH_2CH_2OH)_2$) that reacts with morpholine in 0·1 M-NaOH. Our explanation is supported by the observation that morpholine reacts faster than the *N*-methylpiperazinium ion when choline chloride is used in place of triethanolamine.

Other data in Table 3 for piperidine in 0·1 M-NaOH confirm that nitrosation proceeds readily with N_2O_4 in place of NOCl. The maximum yield of *N*-nitrosopiperidine/mol nitrosyl gas, using 0·05 M-*N*-nitrosodiethanolamine as catalyst, is lower for N_2O_4 than for NOCl (*c.* $4·9 \times 10^{-4}$ and $17·5 \times 10^{-4}$ M, respectively, from 2×10^{-3}M piperidine), but this is consistent with previous estimates of the relative reactivities of NOCl (Challis & Shuker, 1979b) and N_2O_4 (see above). The rates of both reactions, however, are very similar ($t_{1/2}$ about 4 min), which implies that a common nitrosating agent derives from the reaction of either NOCl or N_2O_4 with *N*-nitrosodiethanolamine.

Finally, the comparative effect of 0·5-M quantities of several different alkanolamines, choline chloride and *N*-nitrosoalkanolamines plus $1·14 \times 10^{-2}$ M-NOCl are summarized in Table 3 for reaction with 2×10^{-3} M-piperidine in 0·1 M-NaOH at 25°C. Both the maximum yields of *N*-nitrosopiperidine and its rate of formation (compare $t_{1/2}$ values) are dependent on the catalyst structure, increasing in proportion to the electron-withdrawing capability of the *N*-substituents. These effects are more readily apparent for reaction versus time plots in Fig. 2 for reaction in the presence of *N*-nitrosodiethanolamine, triethanolamine and diethanolamine. Significantly, the best catalyst is *N*-nitrosodiethanolamine which gives $1·71 \times 10^{-3}$ M-*N*-nitrosopiperidine (*c.* 85% reaction) over 38 min. Independent checks established that no *N*-nitrosopiperidine resulted from the interaction of piperidine with either *N*-nitrosodiethanolamine or *N*-methyl-*N*-nitrosoethanolamine in the absence of

Table 3. *Maximum yields and reaction half-lives ($t_{1/2}$) for N-nitrosopiperidine formation from 2×10^{-3} M-piperidine with $1·14 \times 10^{-2}$ M-NOCl and a catalyst (0·05 M) in 0·1 M-NaOH at 25°C*

Catalyst	10^4[*N*-Nitrosopiperidine]† (M)	$t_{1/2}$ (min)
Triethanolamine	12·1	19
Diethanolamine	3·75	24
N-Nitrosodiethanolamine	17·5	4
N-Nitrosodiethanolamine†	4·9	3·5
N-Methylethanolamine	0·60	61
N,*N*-Diethylethanolamine	0·42	100‡
N-Acetylethanolamine	7·51	17
N-Methyl-*N*-nitrosoethanolamine	10·8	10
Choline chloride	10·0	3

*Maximum yield after *c.* 24 hr.
†With $1·0 \times 10^{-2}$ M-N_2O_4.
‡Estimated.

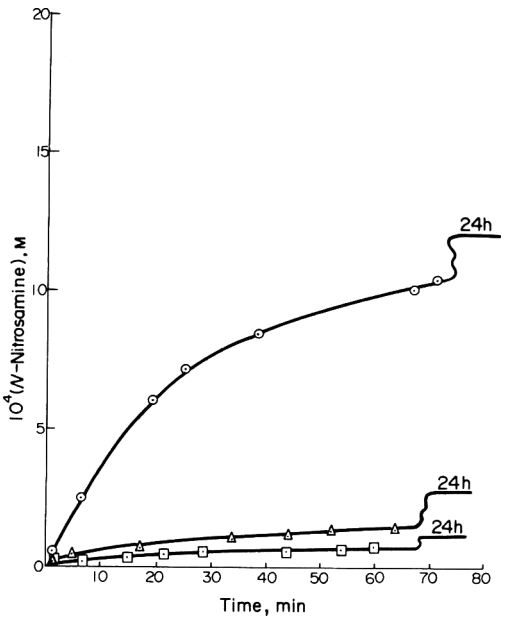

Fig. 1. Effect of amine basicity on *N*-nitrosamine formation from $1{\cdot}14 \times 10^{-2}$ M-NOCl and 2×10^{-3} M-piperidine in 0·1 M-NaOH (⊙) or 2×10^{-3} M-morpholine in 0·1 M-NaOH (⊡) or 2×10^{-3} M-*N*-methylpiperazine in phosphate buffer at pH 6·85 (△), in the presence of 0·05 M-triethanolamine at 25°C.

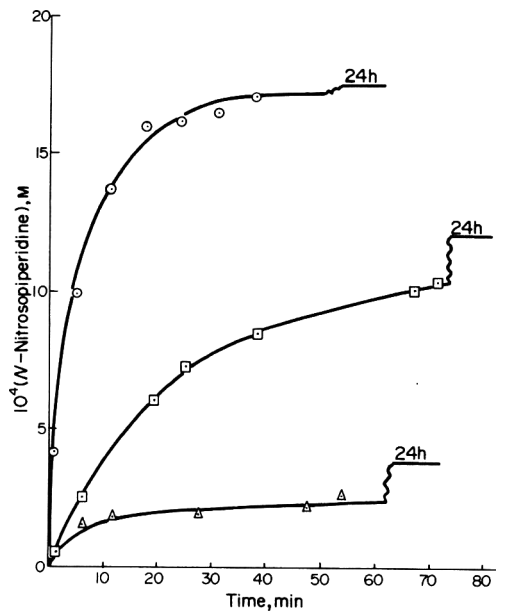

Fig. 2. Variation in *N*-nitrosopiperidine concentration with time for reaction of 2×10^{-3} M-piperidine and $1{\cdot}14 \times 10^{-2}$ M-NOCl in the presence of 0·05 M-*N*-nitrosodiethanolamine (⊙) or 0·05 M-triethanolamine (⊡) or 0·05 M-diethanolamine (△) in 0·1 M-NaOH at 25°C.

nitrosyl gas. Thus, transnitrosation of the *N*-nitroso groups to piperidine does not contribute to the extent of these reactions.

DISCUSSION

There is good evidence that triethanolamine reacts readily with nitrosyl gases and this compound has been recommended for both the removal (Levaggi, Siu, Feldstein & Kothny, 1972) and the estimation of NO_2 (Gold, 1977) in studies of atmospheric pollution. The product resulting from this interaction has not been fully characterized and its properties as a nitrosating agent have not been recognized. Alcohols, however, are well-known to react with nitrosating agents to form alkyl nitrites, as in equation 11, and nitrosyl gases have been shown to be very effective nitrosating agents in organic and in neutral and alkaline aqueous solutions (Challis & Kyrtopoulos, 1978 & 1979; Challis & Shuker, 1979b).

$$ROH + NOX \underset{}{\overset{HO^-}{\rightleftharpoons}} RONO + X^- + H_2O \qquad (11)$$

Alkanolamines are unlikely to be exceptional in either one of these respects, but *N*-nitrosamine formation and deamination, respectively, will compete with alkyl nitrite formation for secondary and primary alkanolamines. Usually, alkyl nitrites are ineffectual nitrosating agents under non-acidic conditions (Allen, 1954), but it has been shown recently (Challis & Shuker, 1979a) that this qualification does not apply to alkyl nitrites bearing electron-withdrawing β-substituents. For example, 2-ethoxyethyl nitrite ($EtOCH_2CH_2ONO$) rapidly converts both piperidine and morpholine to the corresponding *N*-nitrosamines in 0·1 M-NaOH at 25°C and catalysis similar to that reported here applies to 1,2-dihydroxy alcohols (Challis & Shuker, 1979a). These arguments strongly suggest that alkanolamines increase the extent of reaction by initial formation of an alkyl nitrite derivative (I), which then reacts with the secondary amine to give an *N*-nitroso product, in accordance with the following scheme (12):

$$NOX + HOCH_2CH_2NR_2 \overset{HO^-}{\rightleftharpoons} ONOCH_2CH_2NR_2 + H_2O + X^-$$

(I)

$$\xrightarrow{R'_2NH} R'_2NNO + HOCH_2CH_2NR_2 \qquad (12)$$

This mechanism is consistent with equation 10, which also requires that the interaction of amine with I is rate-limiting. For given conditions and reagent concentrations, the ultimate yield of *N*-nitrosamine depends on the selectivity of NOX for alkanolamine and solvent (i.e. ability for form I rather than NO_2^- by solvolysis) *and* the selectivity of I for secondary amine and solvent (i.e. its ability to form *N*-nitrosamine rather than NO_2^-). The rate of *N*-nitrosamine formation, however, is a function of both the reactivity and the selectivity of I. Assuming that 0·05 M-alkanolamine, irrespective of its structure, traps most of the nitrosyl gas, the results in Table 3 qualitatively support these deductions. Thus, both the rate (proportional to $t_{1/2}$) and the extent of *N*-nitrosamine formation increase with increasing electron-withdrawal by the alkanolamine substituents, which should make the alkyl nitrite intermediate, I, more reactive and therefore less selective.

It is clear that powerful nitrosating agents capable of forming *N*-nitrosamines readily under mild, non-acidic conditions are generated by the interaction of alkanolamines and related compounds with nitrosyl gases. Since these reactions apply to N_2O_4 as well as NOCl, and triethanolamine quantitatively absorbs NO_2 from dilute gas streams (Levaggi *et al.* 1972), these reactions probably contribute to the formation of *N*-nitrosamines in consumer products containing alkanolamine emulsifiers.

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STUDIES OF *IN VITRO* CELL TRANSFORMATION AND MUTAGENICITY BY SURFACTANTS AND OTHER COMPOUNDS*

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Abstract—Cryopreserved primary cultures of Syrian golden hamster embryo cells were used as the source of target and feeder cells for an *in vitro* bioassay of carcinogenesis. Three different cultures that gave the best responses in a preliminary test were used for the bioassay. The capacities of ten surfactants and two other compounds to induce morphological transformation were examined using this system. The mutagenic potential of these materials was also tested using *Salmonella typhimurium* strains TA100 and TA98. None of the ten surfactants tested induced *in vitro* transformation of hamster embryo cells, neither were they mutagenic in *S. typhimurium*. Of the two other compounds tested, *N*-nitrosomethyl-*n*-dodecylamine was mutagenic, and dimethylglyoxime induced transformation, the results suggest that neither of these two short-term tests for determining carcinogenicity is adequate alone and that combining the two systems will give more useful results.

INTRODUCTION

It has been estimated that a high proportion of human cancer is caused by exposure to chemicals (Boyland, 1969; Cairns, 1975). Each year more chemicals are added to our environment and to the list of compounds that must be screened for possible carcinogenicity. To evaluate the carcinogenic potential of every environmental and industrial chemical in lengthy and expensive animal studies would clearly be impossible within the foreseeable future. For this reason, various short-term tests for the detection of potential carcinogens have been developed (Montesano, Bartsch & Tomatis, 1976; Stoltz, Poirier, Irving, Stich, Weisburger & Grice, 1974).

Of these, the *Salmonella typhimurium* mutagenesis test developed by Ames has been used the most extensively as an initial screen (Bridges, 1976), and a good correlation has been observed between mutagenicity and carcinogenicity (McCann, Choi, Yamasaki & Ames, 1975; McCann, Springarn, Kobori & Ames, 1975; Sugimura, Sato, Nagao, Yahagi, Matsushima, Seino, Takeuchi & Kawachi, 1976), although some false negatives and false positives do occur. The chemically-induced *in vitro* transformation of mammalian cells also offers considerable promise as a prescreen for chemical carcinogens (Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood, 1976). Recently, a reliable *in vitro* bioassay using cryopreserved primary hamster embryo cells has been developed and shows a high correlation between morphological transformation by chemicals and their reported carcinogenicity (Pienta, Poiley & Lebherz, 1977). These workers found about a 90% correlation between the capacity to induce morphological transformation and carcinogenicity.

Surfactants are incorporated into detergent products, cosmetics, toiletries, drugs and foods widely used by the general consumer, and are also used in almost all industries. In view of the extensive use of these materials their safety has been the subject of many investigations. In addition to the usual acute and prolonged toxicity tests, there have been investigations into carcinogenicity, teratogenicity, absorption and excretion, skin irritation and allergic reaction. Even so not all surfactants used have been tested for carcinogenic effects in animals and little work has been done on their mutagenicity.

The present studies were undertaken to examine the *in vitro* carcinogenic and mutagenic activities of ten surfactants in use on a large scale and two other compounds. These compounds are commercial or synthetic products now in use in industry, with the exception of three cationic surfactants of quaternary ammonium type. The commercial products of these cationic surfactants are usually contaminated by a variety of substances derived from commercial raw materials or processes for producing these compounds. It is important, therefore, to test contaminated samples. Previous results using microbes showed that these commercial products were not mutagenic and did not damage DNA (Inoue & Sunakawa, 1979). The aim of the present study, however, was to obtain comparative data on each of these highly-purified quaternary ammonium surfactants with alkyl chain lengths of C_{16}.

* This work was presented at the 10th IFSCC International Congress, October 1978, Sydney, Australia.

EXPERIMENTAL

Chemicals

Anionic surfactants. Linear alkylbenzene sulphonate (LAS) was prepared by the sulphonation of linear alkylbenzene having a hydrocarbon chain length distribution of C_{10} to C_{14}, and is typical of the alkylbenzenes now in use. After sulphonation, the test material in solution was shown by analyses to be 22·2% active. The impurities were unreacted alkylbenzene, 0·033% and Na_2SO_4, 0·02%. The pH of a 1% solution was 7·1. Sodium alkylpoly(oxyethylene) sulphate was prepared according to the factory specifications. A fatty alcohol, having 53·3% C_{12} and 42·9% C_{13}, was ethoxylated with ethylene oxide and sodium hydroxide to give a product containing an average of 2·5 ethoxylate units per molecule. The ethoxylate was sulphated by reaction with sulphur trioxide, and the resultant acidic product was neutralized and bleached by pouring it into a mixture of sodium hydroxide and sodium hypochloride. The product was an aqueous solution containing 50% $R(OC_2H_4)_{2\cdot5}OSO_3Na$. Impurities were unreacted ethoxylate, 0·9% and inorganic salt, 0·51%.

Cationic surfactants. Cetyltrimethylammonium chloride (CTAC) and cetyldimethylbenzylammonium chloride (CDBAC) were purchased from Wako Pure Chemical Co., Osaka and Tokyo Kasei Co., Tokyo respectively. The CTAC from an acetone–ethanol mixture and the CDBAC from acetone were purified by recrystallization twice. The two samples, CTAC and CDBAC, were shown to be 99·3% and 98·4% pure, respectively. Dicetyldimethylammonium chloride was a gift from Mr. Y. Minegishi of our Tokyo research laboratories, and was shown to be 97·5% pure.

Nonionic surfactants. N,N-Bis(2-hydroxyethyl) lauramide obtained from Kawaken Fine Chemical Co., Ltd., Kawagoe was prepared by the reaction between diethanolamine and an aliphatic carboxylic acid methyl ester (C_{10}, 5%; C_{12}, 85%, C_{14}, 10%). The product contained the active ingredient, 93·9% and water, 1·5%, and its pH was 7·1. Polyoxyethylene sorbitan monostearate (Tween 60) and sorbitan monostearate (Span 60) were commercial products manufactured by Kao Atlas Co., Tokyo.

Amphoteric surfactants. N,N-Dimethyldodecylamine oxide was manufactured by H_2O_2 oxidation of dimethyldodecylamine having a homologue distribution as follows: C_{10}, 0·8%, C_{12}, 97·5%, C_{14}, 1·7%. It was an aqueous solution containing active ingredient, 29·1%, water, 70·6% and H_2O_2, 0·03%. *N,N*-Dimethyltetradecylamine, having 2·6% C_{12}, 96·2% C_{14} and 1·2% C_{16}, was reacted with H_2O_2 to give *N,N*-dimethyltetradecylamine oxide. The product contained the active ingredient, 26·7% and water, 73·3%.

Other compounds. N-Nitrosomethyl-*n*-dodecylamine was prepared in our laboratories, and evidence of its structure was obtained from IR and mass spectra. Its purity (above 98%) was determined on the basis of GLC analysis. Dimethylglyoxime, purchased from Wako Pure Chemical Co., Osaka was of guaranteed reagent grade and was used without further purification.

In vitro *cell transformation assay*

Culture medium and trypsin. The standard complete culture medium used was Dulbecco's modified Eagle medium (No. 188G, Gibco, Grand Island, N.Y., USA) supplemented with 2 mM-L-glutamine and 20% foetal bovine serum (Microbiological Associates, Walkersvill, MD, USA) without any antibiotics. For dissociation of embryos and preparation of subcultures, 0·25% trypsin solution was used; i.e. 10 ml trypsin–EDTA solution (10× No. 540, Gibco) and 90 ml Ca^{2+}- and Mg^{2+}-free solution (PBS).

Preparation of cell cultures. Pregnant Syrian golden hamsters were killed on days 13 and 14 of gestation for preparation of target cells and feeder-layer cells respectively. Embryos without heads and viscera were minced with scissors, and trypsinized with 0·25% trypsin. Inocula of 1×10^7 embryo cells per 75-cm^2 flask were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. When they became confluent primary cultures were trypsinized, dispensed in lots of 5×10^6 cells in glass ampules, and stored in liquid nitrogen for use as target and feeder-layer cells.

Bioassay. The transformation assay was carried out according to the method of Pienta *et al.* (1977) with some modifications (Takayama, Katoh, Tanaka, Nagao, Wakabayashi & Sugimura, 1977). The assay takes 15 days from start to finish. On Day 0, an ampule of cryopreserved primary cells prepared as feeder-layer cells was rapidly thawed and plated in a 75-cm^2 flask containing 20 ml of the culture medium. The medium was changed every day. On day 3, an ampule of cryopreserved primary cells prepared as target cells was also rapidly thawed and plated in a 75-cm^2 flask. On day 4, the feeder cells which were shifting from a stage of logarithmic growth to a stationary phase were irradiated with 5000 R from a linear accelerator (Toshiba LMR-13: Tokyo), trypsinized, and then plated at 6×10^4 cells/60-mm dish in 2 ml of complete medium. On day 5, the target cells which were approximately 80–90% confluent were trypsinized, and a suspension of 500 target cells in 2 ml of complete medium was then added to each of the dishes plated the day before with irradiated feeder-layer cells. On day 6, an appropriate dose of the test chemical in a volume of 4 ml was added, giving a total volume of 8 ml of medium in each dish. Nine dishes were used for each dose level in all the experiments (in a few cases only eight or seven dishes were used for controls). On day 14, the cultures were fixed with absolute methanol for 10 min and stained with Giemsa solution for 45 min or more. The stained dishes were examined with a stereoscopic dissection microscope (Nikon, Tokyo) to count normal and transformed colonies.

Criteria for transformation. Randomly oriented three-dimensional growth with extensive crossing-over of the cells at the periphery of the colony was considered to be the endpoint of morphological transformation (DiPaolo, Nelson & Donovan, 1971; Pienta *et al.* 1977). The centres of transformed colonies usually exhibit dense piling-up of cells. Moreover, these cells usually have an increased ratio of nucleus to cytoplasm, are more basophilic, and are variable in size (Sanford, 1974).

Selection of cell lines. Eight lines of hamster embryo cells were obtained from embryos on day 13 of gestation and stored for use as target cells. Samples of each cell line were tested for their susceptibility to transformation with the potent carcinogen, 3-methylcholanthrene. Secondary cultures were prepared, plated

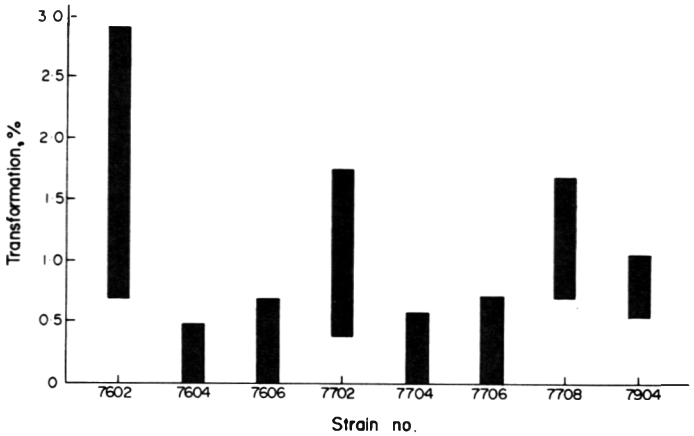

Fig. 1. Morphological transformation by 3-methycholanthrene of cryopreserved hamster embryo cells. Target cell samples in each experiment were derived from one of eight lines of cryopreserved cultures. Cells were treated with graded doses (0·1, 0·5 or 1·0 μg/ml) of 3-methylcholanthrene for 8 days. The length of the bars indicates a range of percentages of surviving transformed colonies corresponding to the range of doses of 3-methylcholanthrene given.

on X-irradiated feeder-layer cells, and treated for 8 days with graded doses (0·1, 0·5 and 1·0 μg/ml) of 3-methylcholanthrene, medium containing 0·2% dimethylsulphoxide or complete medium only.

Mutation test

Salmonella typhimurium strains TA98 and TA100 developed by Ames *et al.* were used as tester strains. The mutation assay was prepared as described by Ames, McCann & Yamasaki (1975) with some modification (Yahagi, 1975). Overnight cultures in nutrient broth were used in all experiments; 0·1 ml of bacterial suspension was added in 0·5 ml of sodium phosphate buffer (0·1 M, pH 7·4) containing the test substance (dissolved in distilled water or dimethylsulphoxide). The contents were preincubated at 37°C for 20 min, and then 2 ml of molten (45°C) top agar was added. In order to incorporate the metabolic activation system, 0·5 ml S-9 mix was added instead of sodium phosphate buffer in a parallel series of experiments. The S-9 mix contained per ml: 200 μl or 300 μl of S-9 fraction (prepared as described by Ames *et al.* (1975) from rats pretreated with polychlorinated biphenyl), 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose-6-phosphate, 4 μmol NADPH and 100 μmol sodium phosphate buffer. The top agar was overlayed on a plate of minimal glucose agar and revertant colonies were scored after 48 hr incubation at 37°C.

RESULTS

Morphological transformation of hamster embryo cells

The results of the tests to select the best cell lines are shown in Fig. 1. Four of the eight samples (7602, 7702, 7708 and 7904) gave responses at all the doses tested. The other four samples (7604, 7606, 7704 and 7706) showed transformation at certain doses. These results indicate that all the batches of cells showed responses, although they varied. In this series of experiments, no transformed colonies were observed in the control groups treated with 0·2% dimethyl sulphoxide or medium only. Since they gave responses at all the doses tested, and an adequate number of cryopreserved samples were available, cultures 7602, 7708 and 7904 were used as a source of target cells for the *in vitro* bioassay of carcinogenesis.

Although a heterogeneous population of colonies is observed in this system, the end-point used for determining the activity is the presence of fibroblast-like colonies morphologically altered beyond the form observed in normal cultures as described in the experimental section.

Induction of transformation by surfactants and other compounds

The results of the transformation assay are shown in Table 1. None of the surfactants or other compounds tested were found to produce transformation at any of the doses tested with the exception of dimethylglyoxime. LAS was fairly cytotoxic at 50 μg/ml. CTAC was very cytotoxic at 5 μg/ml and dicetyldimethylammonium chloride was less toxic. Both Tween 60 and Span 60 were very cytotoxic at 300 μg/ml. Dimethylglyoxime produced transformed colonies at several doses. Spontaneous transformation was not observed in control cultures treated with medium alone or 0·2% dimethyl sulphoxide throughout this series of experiments.

Mutation test

The results of the mutation test are presented in Table 2. The *S. typhimurium* tester strains were periodically checked with known chemical mutagens for their sensitivity. Both strains TA100 (detects base-substitution-type mutations) and TA98 (detects frame-shift mutations) responded positively to 4-nitroquinoline 1-oxide, and strain TA100 also responded to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a mutagen causing base-pair substitutions. The microsomal activation systems were tested with mutagens that are known to require metabolic activation. Benzo[*a*]pyrene and 2-acetylaminofluorene gave positive responses with strains TA100 and TA98 when metabolic activation with rat-liver enzymes was used. A

Table 1. *Transformation of cryopreserved hamster embryo cells by ten surfactants and two other compounds*

Compound	Cell culture	No. of transformed colonies/No. of surviving colonies															
		0·2% DMSO§	3MC§ (μg/ml)			Dose of test material (μg/ml)											
			0·1	0·5	1·0	0§	0·01	0·05	0·1	0·5	1	5	10	20	50	100	300
Anionic surfactants																	
Linear alkylbenzene sulphonate	7708	0/607	1/512	0/438	0/430	0/601	—	—	—	—	—	0/496	0/399	0/371	0/241	—	—
	7708	0/603	0/538	1/527	0/500	0/620	—	—	—	0/617	0/608	0/595	0/544	—	—	—	—
Sodium alkylpoly-(oxyethylene) sulphate	7708	0/607	1/512	0/438	0/430	0/601	—	—	—	—	—	0/508	0/512	0/500	0/408	—	—
Cationic surfactants																	
Cetyltrimethylammonium chloride	7708	0/470†	1/504	0/520	0/490	0/545	—	—	0/588	—	0/413	0/9	—	—	—	—	—
	7708	0/546	0/441	0/410	1/422	0/552	—	0/515	0/499	0/438	—	—	—	—	—	—	—
Dicetyldimethylammonium chloride	7708	0/603	0/538	1/527	0/500	0/620	—	0/582	0/577	0/574	0/566	—	—	—	—	—	—
Cetyldimethylbenzylammonium chloride	7708	0/603	0/538	1/527	0/500	0/620	0/583	0/606	0/576	0/496	—	—	—	—	—	—	—
Nonionic surfactants																	
N,*N*-Bis(2-hydroxyethyl)lauramide	7708	0/607	1/512	0/438	0/430	0/601	—	—	0/535	0/512	0/493	0/483	0/472	—	—	—	—
	7708	0/604	0/569	2/543	0/519	0/609	—	—	0/620	—	0/598	—	0/614	—	—	—	—
	7708	0/546	0/441	0/410	1/422	0/552	—	—	0/528	—	0/537	—	0/553	—	—	—	—
Polyoxyethylene sorbitan monostearate	7904	0/230	1/204	0/202	0/223	0/260	—	—	—	—	0/270	—	0/268	—	0/202	0/200	0/0
	7904	0/329	1/300	3/277	1/252	0/341	—	—	—	—	—	—	0/310	—	0/285	0/220	—
Sorbitan monostearate	7904	0/230	1/204	0/202	0/223	0/260	—	—	—	—	0/237	—	0/229	—	0/198	0/164	0/63
	7904	0/329	1/300	3/277	1/252	0/341	—	—	—	—	—	—	0/310	—	0/258	0/245	—
Amphoteric surfactants																	
N,*N*-Dimethyldodecylamine oxide (C_{12})	7708	0/459‡	0/395†	1/414	1/378	0/520†	—	—	—	—	0/366	0/357	0/357	0/163	—	—	—
	7708	0/592	1/541	2/467†	0/520	0/581	—	—	0/565	0/541	0/552	—	—	—	—	—	—
	7708	0/579	0/409†	2/441	0/432	0/623	—	—	—	—	0/548	0/564	0/502	—	—	—	—
N,*N*-Dimethyltetradecylamine oxide (C_{14})	7708	0/592	1/541	2/467	0/520	0/581	—	—	0/568	0/549	0/558	—	—	—	—	—	—
	7708	0/579	0/409†	2/441	0/432	0/623	—	—	—	—	0/568	0/490	0/192	—	—	—	—
	7708	0/604	0/569	2/543	0/519	0/609	—	—	—	0/594	0/581	0/579	—	—	—	—	—
Other compounds																	
N-Nitrosomethyl-*n*-dodecylamine	7708	0/644	0/585	1/550	1/524	0/652	—	—	—	—	0/681	—	0/670	—	—	0/0	—
Dimethylglyoxime	7602	0/376	2/293	2/227	1/203	0/368	—	—	0/316	—	—	0/294	2/305	—	—	—	—
	7602	0/427	2/263	0/216	0/226	0/462	—	—	2/461	—	2/458	—	3/461	—	—	—	—

DMSO = Dimethylsulphoxide MC = 3-Methylcholanthrene

*In all cases nine dishes each seeded with c.500 target cells were used except where marked with daggers (†eight dishes were used; ‡seven dishes were used).

§Cells treated with dimethylsulphoxide provided the solvent control, 3-methylcholanthrene, the positive control and culture medium alone, the tissue culture control.

Table 2. *Mutagenicity of ten surfactants and two other compounds for* S. typhimurium *strains TA100 and TA98*

Compound	Dose (μg/plate)	No. of His$^+$ revertants/plate with or without S-9 mix using strain			
		TA100		TA98	
		(−) S-9	(+) S-9	(−) S-9	(+) S-9
Controls					
H_2O	—	145	137	19	32
DMSO	—	151	141	23	28
4-Nitroquinoline 1-oxide	0·5	1588	148	167	36
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	2	4152	128	27	30
Benzo[*a*]pyrene	5	178	928	35	614
2-Acetylaminofluorene	50	128	1080	18	2292
N-Nitrosodimethylamine	3000	143	3154	20	34
Linear alkylbenzene sulphonate	10	152	101	38	26
	25	174	105	23	32
	50	190	103	23	24
	100	179	126	22	23
	200	104	118	17	28
Soduim alkylpoly(oxyethylene) sulphate	10	136	130	23	32
	50	130	132	20	31
	100	114	134	12	32
	200	118	134	22	30
	1000	10	117	13	21
Cetyltrimethylammonium chloride	0·05	122	105	12	18
	0·1	121	110	12	30
	0·5	127	123	19	38
	1	105	103	14	22
	5	0*	127	0*	24
	10	0*	133	0*	25
Dicetyldimethylammonium chloride	1	114	129	19	23
	10	148	114	23	30
	50	142	122	15	35
	100	133	141	12	40
	200	129	143	18	30
	400	130	125	15	32
Cetyldimethylbenzylammonium chloride	0·01	123	139	12	29
	0·05	133	108	13	22
	0·1	105	120	10	40
	0·5	100	115	17	18
	1	96	129	15	26
	5	0*	132	0*	28
	10	0*	91	0*	18
N,*N*-Bis(2-hydroxyethyl)lauramide	10	120	118	23	31
	50	110	105	18	29
	100	130	126	19	27
	200	37	113	11	30
	1000	0	91	0	18
Polyoxyethylene sorbitan monostearate	10	121	111	18	31
	100	134	127	23	29
	200	157	133	33	34
	1000	148	141	22	33
	2000	115	136	24	33
Sorbitan monostearate	10	137	133	17	25
	100	131	123	14	36
	200	150	134	22	32
	1000	133	142	16	30
	2000	130	157	17	30
N,*N*-Dimethyldodecylamine oxide	10	134	141	20	31
	25	131	137	25	35
	50	140	116	25	26
	100	116	140	12	28
	200	0*	145	0*	39
N,*N*-Dimethyltetradecylamine oxide	10	123	155	22	29
	25	113	140	23	28
	50	112	120	27	30
	100	60	159	16	36
	200	11	140	14	27

(*Continued on next page*)

Table 2. (*Continued*)

Compound	Dose (μg/plate)	No. of His$^+$ revertants/plate with or without S-9 mix using strain			
		TA100		TA98	
		(−) S-9	(+) S-9	(−) S-9	(+) S-9
N-Nitrosomethyl-*n*-dodecylamine	50	123	158	21	20
	100	112	206	23	26
	200	62	360	11	33
	300	63	432	13	27
	500	40	314	5	21
	1000	NT	180	NT	28
	3000	NT	142	NT	20
	5000	NT	148	NT	14
Dimethylglyoxime	10	118	174	16	31
	50	133	158	23	27
	100	157	143	20	31
	500	135	141	12	36
	1000	132	133	16	28
	2000	152	149	20	33

NT = Not tested DMSO = Dimethylsulphoxide

*The compound was particularly toxic to the bacteria at these doses.

similar positive response was noted for *N*-nitrosodimethylamine with strain TA100.

None of the ten surfactants assayed showed any mutagenic activity against TA100 and TA98 either with or without activation. Of the two compounds, *N*-nitrosomethyl-*n*-dodecylamine showed significant but low mutagenic activity using TA100 after metabolic activation. Dimethylglyoxime was not mutagenic towards either strain.

DISCUSSION

There are now various short-term screening tests for the identification of environmental carcinogens (Bridges, 1976; Montesano *et al.* 1976; Stoltz *et al.* 1974). Of these, it has been indicated that the *Salmonella*/microsome mutagenicity test and an *in vitro* cell transformation assay are both reasonably efficient at predicting carcinogenicity in animals. Although none of these tests can replace long-term tests in animals, they are of great value in establishing priorities for long-term testing.

Two promising cell systems have been developed as *in vitro* models for the study of chemical carcinogenesis: one system utilizes heterogeneous primary or secondary cultures of hamster embryo cells (Berwald & Sachs, 1963 & 1965; DiPaolo, Donovan & Nelson, 1969; DiPaolo, Nelson & Donovan, 1969; DiPaolo *et al.* 1971; Huberman & Sachs, 1966; Kuroki & Sato, 1968), while the other uses clonable long-term lines of mouse fibroblasts (Chen & Heidelberger, 1969a,b,c; DiPaolo, Takano & Popescu, 1972; Kakunaga, 1973; Marquardt, Sondergren, Simp & Grover, 1974; Mondel & Heidelberger, 1970; Reznikoff, Bertram, Brankow & Heidelberger, 1973). In this study, we used cryopreserved primary cultures of Syrian golden hamster embryo cells as the source of target and feeder layer cells for *in vitro* carcinogenesis bioassay (Pienta *et al.* 1977). It was demonstrated that target cells freshly prepared from pools of 13-day-old hamster embryos vary in their susceptibility to a known carcinogen, 3-methylcholanthrene (Fig. 1). Pienta *et al.* (1977) found that about one-half of the cells from eleven secondary cultures were not transformed by 3-methylcholanthrene at any of the doses tested, a further indication that not all samples of cells are susceptible to transformation. We found that all the samples of freshly prepared early passage hamster embryo cells showed responses, although they varied among different pools. In the present work we used three different cultures giving the overall responses in a previous test to minimize non-responsiveness with cells.

Of the ten surfactants tested, three surfactants, namely LAS (Bornmann & Loeser, 1963; Buehler, Newman & King, 1971) sodium alkylpoly(oxyethylene) sulphate (Tusing, Paynter, Opdyke & Synder, 1962) and CDBAC (Alfredson, Stiefel, Thorp, Barten & Gray, 1951; Fitzhugh & Nelson, 1948), have been found to be non-carcinogenic in animal experiments. Although CTAC has not been tested for carcinogenic effects, its analogue, cetyltrimethylammonium bromide (Isomaa, Reuter & Djupsund, 1976) had none in rats. These four surfactants did not induce *in vitro* transformation of hamster embryo cells neither were they mutagenic. The two anionic surfactants, Tween 60 and Span 60, gave negative results for transformation and mutagenicity. It was reported that these surfactants were not carcinogenic when they were fed to mice, rats, hamsters or dogs (Brush, McCoy, Rosenthal, Stauber & Allison, 1957; Fitzhugh, Bourke, Nelson & Frawley, 1959; Oser & Oser, 1957). However, it has been reported that Tween 60 is a weak promoting agent (Setälä, 1956; Setälä, Setälä & Holsti, 1954) and a weak carcinogen (Della Porta, Shubik, Dammert & Terracini, 1960; Shubik, Della Porta & Spencer, 1959) after topical application to mouse skin, and also produces local sarcomas after sc

injection in rats (Lusky & Nelson, 1957). It was observed that tumour incidences were within control ranges, when its carcinogenicity was tested by four consecutive sc injections in infant Swiss mice (Epstein, Fujii, Andrea & Mantel, 1970). Tween 60 gave negative results in the majority of screening tests for the detection of carcinogens (Odashima, 1976) and our results on mutagenicity to *S. typhimurium* agree with this. With respect to the skin carcinogenesis it has been suggested that since the quantity of Tween 60 that must be applied to the skin in an individual application is so large, it seems possible that this is a gross physical phenomenon perhaps quite different from the action of other chemicals in skin carcinogenesis. The four surfactants for which we had no animal carcinogenicity data, namely dicetyldimethylammonium chloride, *N,N*-bis(2-hydroxyethyl)lauramide, *N,N*-dimethyldodecylamine oxide and *N,N*-dimethyltetradecylamine oxide, had no mutagenic effects, and did not elicit transformation.

Dimethylglyoxime elicited morphological transformation in two consecutive experiments, but was not mutagenic in *S. typhimurium*; it is a candidate for further evaluation in animal experiments. *N*-Nitrosamines comprise a group of chemicals that are very potent carcinogens for various species of animals. *N*-Nitrosomethyl-*n*-dodecylamine, expected to be carcinogenic by analogy with *N*-nitrosodimethylamine (NDMA), gave a positive response with strain TA100 when metabolic activation with rat liver enzyme was used, but was a weaker mutagen than was NDMA. Transformation was not induced by *N*-nitrosomethyl-*n*-dodecylamine. The results obtained by Pienta *et al.* (1977) showed that early passage hamster embryo cells metabolize many carcinogens to their active forms, but there are a few carcinogens that may not be activated, such as 2-acetylaminofluorene (AAF) or *N*-nitrosodiethylamine. AAF transformed cells when tested in the presence of hamster liver microsomes. Therefore,transformation might be induced if *N*-nitrosomethyl-*n*-dodecylamine were tested in this system using the appropriate microsomes for its activation.

In this study, dimethylglyoxime was the only test compound giving a positive result in the cell transformation assay and was also the only compound tested using cell line number 7602. All other compounds were tested using either cell line 7708 and/or line 7904, and gave negative results. Of these three cultures, 7602 was the most sensitive to transformation. Using this cell line, 7602, the capacities of various compounds to induce morphological transformation have been examined (Takayama, Katoh, Hirakawa & Tanaka, 1978; Umezawa, Matsushima, Sugimura, Hirakawa, Tanaka, Katoh & Takayama, 1977) and some of these compounds gave negative results. In addition, certain other surfactants and other compounds (K. Inoue & S. Takayama, unpublished data 1978 & 1979) have been assayed using cell cultures 7708 and 7904. Both cultures were transformed by these surfactants and compounds. 3-Methylcholanthrene gave low levels of transformation in both 7708 and 7904 and this result was thoroughly reproducible. Therefore, each of these cultures also seems to be a reliable source of target cells for *in vitro* bioassay of carcinogenesis.

These results suggest that neither of these two short-term tests for determining carcinogenicity is adequate alone and that combining the two systems will give more useful results.

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SHORT PAPER

VOLATILE NITROSAMINES IN HUMAN BLOOD BEFORE AND AFTER INGESTION OF A MEAL CONTAINING HIGH CONCENTRATIONS OF NITRATE AND SECONDARY AMINES

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Abstract—Samples of human blood taken before and after ingestion of a Japanese meal containing high concentrations of nitrate and secondary amines were analysed for volatile nitrosamines. Nitrosodimethylamine was detected in all 32 blood samples from eight donors in amounts ranging from a trace (<0·5 ng/ml) to 1·3 ng/ml. The ingestion of the test meal had no clear effect on the blood levels of nitrosamines during the subsequent 2 hr. The results of this study provide some support for the possibility of nitrosamine formation in the lower gastro-intestinal tract.

Introduction

There have been several reports of the presence of carcinogenic *N*-nitroso compounds in the human body. Fine, Ross, Rounbehler, Silvergleid & Song (1979) detected volatile nitrosamines in human blood and reported that the levels of nitrosamines in the blood increased after ingestion of a meal. Nitrosamines have also been detected in human urine (Kakizoe, Wang, Eng, Furrer, Dion & Bruce, 1979) and faeces (Wang, Kakizoe, Dion, Furrer, Varghese & Bruce, 1978) and the possibility of their endogenous formation was suggested. Nitrite, one of the precursors of nitrosamines, is known to be formed from nitrate in human saliva (Ishiwata, Boriboon, Nakamura, Harada, Tanimura & Ishidate, 1975; Spiegelhalder, Eisenbrand & Preussmann, 1976; Tannenbaum, Weisman & Fett, 1976) and also to be present in human intestines and faeces (Tannenbaum, Fett, Young, Land & Bruce, 1978). Foods and water seem the most likely sources of nitrate and amines. Foods with high concentrations of nitrate or amines, such as vegetables, dried fish or fish roes are often used in the Japanese diet. In order to assess the effects of the Japanese diet on the levels of nitrosamines in the blood, a meal containing high levels of nitrate and secondary amines was consumed by Japanese volunteers and the levels of nitrosamines were studied in the blood samples taken before and after the meal.

Experimental

Blood sampling. Blood samples were taken from eight male donors, four (A–D) being 17 years old and four (E–H) 20 years old. From each donor a sample was taken 30 min before the meal and three samples were taken after the meal at various intervals up to 120 min. A 20-ml blood sample was taken at each time and frozen immediately after addition of sodium heparin solution.

Test meal. The midday meal for the experiment consisted of rice, roasted mackerel, roasted cod roes, pickled radishes, dried strips of radishes, soup containing fish balls and salted Chinese cabbage. All donors abstained from foods with high levels of nitrate and secondary amines at supper on the day before the experiment and took only bread and milk for breakfast on the test day.

Nitrosamine analyses. Blood and the test meal were analysed for volatile nitrosamines by a modification of the method of Howard, Fazio & Watts (1970). Sulphamic acid solution was added to the frozen sample (20 ml or 20 g) and then steam distillation was performed in the presence of 10 g KOH and 30 g NaCl. The distillate (150 ml) was trapped in 10 ml N-HCl and then extracted three times with dichloromethane (100 ml each time) after addition of 20 g K_2CO_3. The dichloromethane extracts were dried over anhydrous sodium sulphate and evaporated under vacuum to a volume of 1 ml. Volatile nitrosamine concentrations in the extracts were determined by gas chromatography with a Thermal Energy Analyzer (Thermo Electron Corp, Waltham, MA, USA) as detector (GLC–TEA). A glass column (1·5 m) packed with 10% Carbowax 20 M on Gaschrom P, 60–80 mesh, was used. With this method, the recoveries of nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA) and nitrosopyrrolidine (NPYR) in concentrations of 40, 90 and 80 ng added to 20 ml blood were found to be 44, 58 and 43%, respectively. The determination limit of each volatile nitrosamine in the blood was approximately 0·5 ng/ml when 10 μl of sample solution was injected onto the gas chromatograph. The sample with the highest concentration of NDMA was also estimated by gas chromatography coupled with high-resolution mass spectrometry (GLC–MS). Several samples were treated with HBr in glacial acetic acid according to the method of Eisenbrand (1972) and then applied to the GLC–TEA.

Amine, nitrite and nitrate analyses. Secondary amines in food samples were extracted with a mixture

of N-HCl and ethanol and then determined as dimethylamine (DMA) by a modification of the dithiocarbamate method of Dowden (1938). For the determination of nitrite and nitrate in the meal, samples were deproteinized by the method of Wegner (1972) with some modifications by M. Harada (personal communication, 1975) and were homogenized in 10-g quantities with hot water and heated at 80°C for 20 min after addition of 10 ml 0·5 N-NaOH and 10 ml 12% $ZnSO_4$. The solution was filtered after addition of 20 ml 10% ammonium acetate buffer, and the filtrate was analysed for nitrite and nitrate. Nitrite in the filtrate was determined by colouration with Griess reagent; nitrate in the filtrate was reduced to nitrite by passage through a cadmium column (Schall & Hatcher, 1968) and then determined in the same way.

Results and Disscussion

Table 1 shows the levels of volatile nitrosamines found in the blood samples. Levels less than 0·5 ng/ml blood are represented as a trace. NDMA was detected in all 32 blood samples from the eight donors, at levels ranging from trace amounts to 1·3 ng/ml. NDEA was detected in only one sample, from donor C, and only at a trace level. No NPYR was found in any of the samples.

Table 1. *Volatile nitrosamines in blood samples taken before and after ingestion of the test meal*

Donor	Sampling time (min before (−) or after (+) meal)	Level of NDMA (ng/ml blood)
A	−30	0·7
	+30	0·7
	+60	0·5
	+120	0·8
B	−30	0·7
	+30	1·0
	+60	0·8
	+120	Tr
C	−30	1·3
	+30	0·6
	+60	1·1
	+120	0·9
D	−30	0·7
	+30	0·9
	+60	0·8
	+120	0·6
E	−30	1·3
	+10	Tr
	+30	0·7
	+60	Tr
F	−30	Tr
	+10	0·7
	+30	Tr
	+60	Tr
G	−30	Tr
	+10	0·9
	+30	Tr
	+60	Tr
H	−30	Tr
	+10	0·8
	+30	0·9
	+60	Tr

NDMA = Nitrosodimethylamine Tr = Trace

Nitrosopyrrolidine was not detected in any sample and nitrosodiethylamine was limited to a trace in one (pre-meal) sample, taken from donor C.

The reagents and chemicals used in the analytical procedures contained no detectable amounts of volatile nitrosamines. NDMA in the blood sample taken from donor C 30 min before the meal was confirmed by GLC–MS. The level of NDMA determined by GLC–MS was 1·6 ng/ml, similar to that determined by GLC–TEA (1·3 ng/ml). Further, when the samples treated with HBr and glacial acetic acid were applied to the GLC–TEA, they gave no comparable peaks. Therefore, the peaks demonstrated by GLC–TEA are considered to be derived from nitrosamines. The blood levels of NDMA as a function of time varied among individuals. In some cases, NDMA levels increased after the meal, but in other individuals they were higher before than after the meal. The amounts of nitrate, nitrite, secondary amines (as DMA nitrogen) and NDMA in the meals taken by donors A–D were 235·5, 3·0, 8·8 and 0·0017 mg respectively, while in those taken by donors E–H they were 222·8, 2·2, 5·3 and 0·0031 mg, respectively. NDEA and NPYR were not detected in the meals. The kinds of secondary amines in the ingested meal were analysed by gas chromatography after nitrosation and were found to be mainly DMA.

Although the levels of nitrate and secondary amines in the ingested foods were relatively high, the results did not show any clear effects of the meal on the levels of nitrosamines in the blood, at least for periods up to 2 hr after the meal. It was not clear whether the NDMA found in the blood after the meal was derived from the ingested foods or not, because NDMA was also present in the blood before the meal.

In these experiments, the donors had abstained from food containing high concentrations of nitrate and secondary amines for relatively long periods before ingestion of the test meal, but NDMA was nevertheless detected in all blood samples before the meal. Consequently, nitrosamines may exist constantly in the blood. The main sources of nitrosamines present in the blood could be (1) nitrosamines contained in foods, (2) *in vivo* formation in the stomach (Sander & Seif, 1969) and (3) *in vivo* formation in the intestine. It seems unlikely, however, that *N*-nitroso compounds present in foods or produced in the stomach remain for long periods after ingestion of foods (Rounbehler, Ross, Fine, Iqbal & Epstein, 1977). The possible formation of *N*-nitroso compounds by bacteria in the intestine was pointed out by Tannenbaum, Archer, Wishnok & Bishop (1978), and Wang *et al.* (1978) also suggested that nitrosamines present in human faeces, urine and blood were derived mainly from nitrosamines formed in the intestine. Our results similarly suggest the possibility of nitrosamine formation in the lower gastro-intestinal tract.

The levels of NDMA in the blood in this report are higher than those reported by Fine *et al.* (1977), who found a maximum level of approximately 0·8 ng/ml. It is possible that our finding is related to the daily intake of a Japanese diet containing high concentrations of nitrate and secondary amines.

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Review Section

CIIT DEDICATION: ADDRESS OF WELCOME AND INTRODUCTION OF THE MAIN SPEAKER

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(*Received 12 December 1979*)

Lieutenant-Governor Green, Ladies and Gentlemen:

I have the very pleasant task of welcoming you to CIIT. On behalf of the Board of Directors and staff of CIIT, may I say how grateful we all are that so many distinguished figures from the worlds of Academia, Government and Industry are able to be present here today.

You find us in our own facility at last. Pleasure over these beautiful surroundings, and relief that the long gestation period is over, must however be tempered by impatience to start full-scale experimental work here. Shakespeare has told us how "sweet are the uses of adversity", to which I would point out that elsewhere he himself stated that "a surfeit of the sweetest things, the deepest loathing to the stomach brings". There has to be some limit to tolerable adversity! Constant use of superlatives and hyperbole has debased our linguistic currency. The expression 'birth pangs' is an example, much used by men who could not—neither physiologically nor pathologically—experience what birth pangs are really like. Nevertheless, on the basis of a vivid imagination, I can tell you that what we have gone through since occupying this building is about as close as a male *Homo sapiens* can come to being delivered of a full-term foetus with a breech presentation. In this case, it also transpired that, although the offspring was well-formed externally, it was grossly underdeveloped or even malformed with respect to some vital internal systems—such as blood circulation, respiration and digestion, to name but a few. Since hope ever springs eternal, we keep thinking the worst is behind us. All I would ask from you is your understanding and indulgence over any imperfections you encounter in the course of your tour of the laboratory. Please make a mental note to return one day when we have everything running smoothly—we do believe that day is coming!

Speaking seriously, we are really very proud of this building, which incorporates much advanced design and technology, with all that that implies in terms of headaches. Chief credit and thanks for this achievement and—to be fair—for some of these headaches, goes to the staff of CIIT. As each has come on board, he or she has helped in creating this state-of-the-art facility. I am particularly grateful to my two colleagues Dr. James Gibson and Dr. Donald Hart. Without the former, the building could not have been started. Without the latter, I am convinced that it would not have been completed. The participation of so many people inevitably brings to mind the definition of a camel as a horse designed by a committee. I hope that what we have here combines the style, elegance and pace of an Arab steed with the stoic endurance and persistence of the ship of the desert.

We anticipate that the laboratory will make possible the high standard of scientific achievement that we have set for ourselves. The term 'centre of excellence' is not one to bandy about light-heartedly. Our use of this term as a goal for CIIT is an earnest of our dedication to outstanding quality of scientific endeavour, in all facets of Toxicology with which we are concerned. Some have called us 'élitist'. Pejorative though that term may seem, in fact nothing more than an expression of arrogance and snobbery, élitism can connote dissatisfaction with the *status quo* and a desire to strive for the heights in order to attain scientific understanding. I recall a large sign mounted on the wall of a microbiological laboratory in which I once worked. It read "Sterile enough is not sterile enough". As practitioners of Science in the Public Interest, all of us at CIIT feel that only our best is good enough.

I was recently taken with a statement by Nobel Laureate A. T. James concerning three pieces of unconventional wisdom learned from his mentor, colleague and fellow Nobelist, A. J. P. Martin:

(1) Nothing is too much trouble provided someone else does it;
(2) Never answer the first letter; if it's important they'll write again;
(3) If there are twelve ways of tackling a problem, they're all wrong.

As to the first precept, I have always believed in it but could not bring myself to apply it. The second I shall take to heart, starting tomorrow. The third doesn't hold for Toxicology, where all twelve approaches may well provide relevant, even essential information for the purpose of risk assessment of a chemical agent. Like blind men palpating an elephant, we often need to feel out as much as we can in order to grasp the dimensions and topography of the problem. Nonetheless, that precept does have a message

for the scientist concerning the need to eschew mere data-gathering and to define the crux of a problem in order to seek out the unique route to its solution.

All of us at CIIT feel that we are indeed fortunate and privileged to be participating in research that has potential to contribute to fundamental understanding while at the same time serving such important social needs and objectives. The chemical industry deserves credit for affording us this opportunity.

One does not have to be an assiduous reader of the scientific literature to discern the dramatic shifts which the science of Toxicology is undergoing in every aspect of its philosophy and practice. The *Washington Post*, the *New York Times*, the *New Yorker*, *Playboy Magazine*, and even the muted voice of the *National Observer* all convey the same message. Emphasis on environmental pollutants, particularly actual or potential carcinogens, is also reflected increasingly on radio and television. The glare of public scrutiny is focused on the practitioners of Toxicology, while controversies over unresolved and seemingly unresolvable problems swirl about them.

Amidst all this furore, one publication is proving more and more successful in presenting the facts in a timely and balanced fashion. *Science* magazine provides the scientific community, and the world at large, with an objective analysis of controversial and complex issues. The members of the American Association for the Advancement of Science, together with thousands of other readers in 140 countries, have ample cause to be grateful to the distinguished Editor of *Science*, Dr. Philip Abelson. We at CIIT are particularly pleased that he has consented to deliver the Dedication Address* today.

One man in his time plays many parts. Our speaker is a good example of this aphorism. He is a Physical Chemist who has made brilliant contributions to Biophysics and Organic Geochemistry and who has wandered into so many fields of Science that it was inevitable that, sooner or later, he would discover the fascination of Toxicology. He gained his Ph.D. in Nuclear Physics at the University of California in 1939. In the course of a career in Physics, he rose to the position of Principal Physicist at the Naval Research Laboratory in Philadelphia. Then he returned to the Carnegie Institute in Washington first as Chairman of the Biophysics Section, Department of Terrestrial Magnetism (1946–1953), then as Director of the Geophysics Laboratory (1953–1971), and, finally, as President of the Institute from 1971 until 1978. War-time activities included membership of the National Defense Research Committee (1940–1942) and Chairmanship of the Radiation Cataract Committee of the National Research Council (1949–1957).

Literary endeavours such as membership of the Advisory Board of the Journal of the National Cancer Institute and co-editorship of the Journal of Geophysical Research culminated in his appointment as Editor of *Science* in 1962. In the years that followed, he has published volumes on *Geochemistry* (1959 and 1967), *Energy: Use, Conservation and Supply* (1974), *Materials: Renewable and Non-renewable* (1976), *Electronics: The Continuing Revolution* (1977), and, amazingly, *Food: Politics, Economics, Nutrition, and Research* (1975). His Editorials, appearing almost weekly in *Science* are pithy, yet perceptive and attuned to current thought. As they are under scrutiny by the vast and varied membership of AAAS, as well as the worldwide readership of *Science*, it is readily apparent that to satisfy everyone would be a minor miracle—yet somehow or other Dr. Abelson manages to do this, week in and week out. As one of the erstwhile sceptics, I have been most impressed with the changes he has brought about to enable his readers to keep in touch with so many aspects of science, particularly the Washington scene from which emanates so much sweetness and light, or gloom and despair, depending on one's point of view!

As befits a notable public figure, Dr. Abelson has had a fair share of honorary degrees, medals and other distinctions. They are too numerous to list but fittingly reflect his scientific stature. His presence here today not only honours us but adds what I hope he will regard as yet another distinction, as well as the opportunity to address us on a subject of great public importance. His broad range of experience has helped to prepare him for an active role in the public debate on toxic hazards in the environment. In his case, the past is truly prelude to the issues that our speaker will discuss today.

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*Dr Abelson's address, entitled 'New Directions in Toxicology', constitutes the next article in this section (p. 303).

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NEW DIRECTIONS IN TOXICOLOGY*

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(*Received 12 December 1979*)

Today we celebrate an important event in the life of the Chemical Industry Institute of Toxicology. This new building is the tangible outcome of co-operative effort and statesmanship on the part of leaders of the chemical industry. These leaders recognized the need for in-depth studies of the commodity compounds that form the great part of chemical raw materials and products. They recognized that a unified approach to the study of potential effects of chemicals would avoid excessive duplication. They recognized the importance of establishing an independent laboratory charged with exemplifying the highest professional standards.

Toxicology, as it is conducted in most places, is a terribly dull topic. In contrast, at this Institute it is an exciting frontier of biology. In most places the animals are looked on as black boxes. One administers a series of doses and after a time the animals have either lived or died. They have either developed evidence of pathology or they have not. In this Institute the animals are looked on as complex and highly interesting creatures carrying on fascinating chemical conversions and concentrating or excreting the doses or their products in unexpected ways. The Institute is devoting about 60% of its efforts to conventional testing, but about 30% goes to discovery of the underlying phenomena. Thus there is close interaction of testing, pharmacology, biochemistry, histology and pathology, with each reinforcing the others. The intellectual climate is further improved by the presence of pre- and post-doctoral fellows, 10% of the budget being devoted to such training. The programme fills a national need, for there is a shortage of competent toxicologists. The policy of the Institute is to train for 2 years and then to let go. Thus a steady stream through the Institute of new, vigorous young people is assured.

In emphasizing quality rather than quantity the founding leaders were particularly wise. Today, every major company producing chemicals has a toxicology laboratory about the size of the new one here. In the federal government 37 departments have activities related to toxic substances and the annual expenditures total some $600 million. In addition, the National Institute of Environmental Health Sciences is scheduled to have a laboratory costing about $120 million. Thus, the Chemical Industry Institute of Toxicology with its $8 million annual budget will represent a small fraction of a very large activity. Nevertheless, it can become the world's leading centre of toxicology.

Located in the Research Triangle near three fine Universities and many research establishments, this Institute enjoys unusually stimulating surroundings. With a staff of about 100 including 60 professionals drawn from many disciplines, the Institute is off to a good start and is already enjoying a fine internal climate for interdisciplinary research. Facilities and equipment are comprehensive.

In formulating the rules under which this Institute operates, the founders realized that they must give it both financial stability and maneuvering room. The Institute is supported by 34 of the leading companies that manufacture chemicals. Together they represent 85% of the dollar volume of production in the United States. Member companies contribute annually on the basis of a formula related to their sales. A crucial feature is a 3-year rolling commitment. Thus the Institute is assured that in the unlikely event of financial curtailment changes would be gradual.

In the 1977 Annual Report of the Chemical Industry Institute of Toxicology, Chairman of the Board, Richard Fleming, outlined what he and others who had planned this Institute hoped it would achieve. The goals were:

(1) To provide a sound and forceful scientific presence in the complex area of chemical safety environment;
(2) To generate and interpret data on chemicals from the viewpoint of safety to man and the environment;
(3) To development new, improved and faster test methods for assessing the potentially harmful effects of chemicals;
(4) To assess potential human risks involved in manufacturing, handling, using, and disposing of chemicals;
(5) To furnish the scientific expertise essential to evaluate properly the benefit/risk equation;
(6) To disseminate information on potential hazards of chemicals, and to encourage use of such knowledge to minimize human risks;
(7) To promote the professional development and training of toxicologists, epidemiologists and other scientists in related fields.

One of the areas in which the supporting companies have had a voice is in the choice of chemicals to be tested. But that influence in no way detracts from the programme. Today, about 43,000 chemicals are

*Address given at the dedication of the Laboratory of the Chemical Industry Institute of Toxicology at the Research Triangle, North Carolina, 12 September 1979.

manufactured and distributed, some in quantities of less than 1000 pounds a year. Obviously, a programme aimed at studying many of them at once would necessarily be superficial. It is desirable to choose a limited number of important large volume compounds and to study them well. Choice of the specific ones are based on criteria that include the following:

(1) The chemicals are widely used and are the basis for a large number of compounds;
(2) Gaps in toxicological knowledge exist for these chemicals;
(3) Long-term study of these chemicals is expected to make a significant contribution to the science of toxicology.

On the basis of these factors the first priority list of eleven chemicals was established in 1976. These were: aniline, chlorine, dinitrotoluene, ethylene, ethylene oxide, formaldehyde, *n*-hexane, maleic anhydride, methylene chloride, phenol and toluene. Since then 29 others have been added.

The founders have been careful to emphasize that the Institute is to be intellectually independent. The sponsoring members will co-operate in every way and will furnish data and recommendations about chemicals to be tested. However, the President of the Institute and his staff formulate the programme and have complete authority to carry it out. When results are released, they are distributed broadly with no prerelease for insiders. The policy is to submit reports to journals using peer review. The Institute will avoid advocacy roles.

A favourable factor in the future of this Institute is the quality of its President, Leon Golberg. He is widely respected and is knowledgeable in many fields relevant to toxicology. He is especially expert in biochemistry and organic chemistry and pharmacokinetics. His judgement as to the opportunities in the emerging, new toxicology will help to inspire this Institute to revolutionary achievements in the comprehension of biological processes. His command of the literature is excellent and he writes well. He is a statesman whose opinions will come to have even more weight as this institution proves creative and useful. His remarks on the occasion of an address to the Anglo-American Conference on Human Health and Environmental Toxicants were well chosen. He reviewed comprehensively new experiments in toxicology. Some he found substantive, others he found to be poorly conducted and possibly spurious. Some of the latter had been given publicity far in excess of merit. Commenting on the fashion of announcing great discoveries on the basis of little evidence, he wrote:

> "The first danger of new discoveries in Toxicology is excessive haste to protect public health, a headlong rush that may well entail replacement of a well-known material, with a long history of human exposure without any observed adverse effects, by an untried substitute whose own potential for toxicity is largely unexplored. In the US, we have a long tradition of excessive and often ill-balanced reaction of this sort.
>
> "Such experiences suggest that it is imperative to seek out past mistakes and to learn from them, not to bury them. We need to redress the unfortunate imbalance created by undue emphasis on carcinogenicity, to the exclusion of other potential toxic hazards, many of them also long-term, also irreversible, and sometimes less readily treated or cured than cancer. Holism requires consideration of all factors that enter into the disturbance of the dynamic harmony embodied in the concept of homeostasis. To ignore air, water, diet and lifestyle, the hormonal and other modulating factors, and the protective agents and mechanisms—both outside and within the body, when making an assessment of risk to man, ensures an incomplete and distorted view of reality."

Thus far I have been very positive in my remarks about the Institute and its sponsors. I turn now toward two aspects about which I have some slight reservations. One is the problem of establishing and maintaining credibility for the Institute. The second is the matter of instrumentation. I have discussed this Institute with persons of good will, who have raised questions about credibility. Their attitudes reflect a widespread suspicion of business and specifically of the chemical industry. The founders have made a substantial effort to anticipate scepticism. One example of their efforts to counter scepticism is the policy of peer-reviewed publication of results with no favouritism in release to sponsors. Already the federal government designates three members of the Scientific Advisory Committee. That is a good step, but the Institute should be encouraged to go even further. That is, a devoted but constructive environmentalist might be asked to serve on the Advisory Committee. There could be other measures. Indeed, environmentalists might be consulted for suggestions. In any event the most precious objectives that the Institute can achieve are credibility and excellence.

To attain excellence in the toxicology of tomorrow, an institute must have first class personnel and it must be superbly equipped. This Institute has good equipment and its analytical facilities match or far surpass those of other toxicological laboratories. However, the analytical capabilities do not match those I have seen in the great industrial research establishments. Many of the companies that are sponsoring this laboratory have magnificent analytical facilities which are ahead of the common state of the art. They have staffs whose function is to maintain leadership in the fast moving field of analytical methods and instrumentation. Abilities to detect extremely tiny amounts are reaching fabulous sensitivities. In some instances measurements have been made at the level of 10^{-14} grams. Such sensitivities are far more than adequate to permit unprecedented observations of pharmacological mechanisms. For example, it would be possible to illuminate one of the great controversies of toxicology, that is, response to low doses. Another opportunity would be observations on humans who have inadvertently been exposed to very low levels of chemicals in the workplace.

This Institute cannot maintain the size and kind of staff of analytical experts that the large industrial laboratories enjoy. However, it should be feasible for the Institute to obtain valuable advice and in some

instances measurements from the experts. I recommend urgently that this Institute and its sponsors consider how best to implement a good interaction between the analytical experts of the companies and the staff here.

I turn now to a further discussion of the role of this Institute among other laboratories in the field of toxicology. This is a rapidly expanding field, with work being carried on in contract laboratories, industry, government and academia. The many industrial laboratories concentrate their efforts on testing proprietary products. Their objectives are principally to determine toxic levels. Little time or effort is available to conduct research into pharmacological mechanisms. The same is true of the contract laboratories. In general the universities do not have facilities to conduct complete testing programmes. With some exceptions, their output has tended to emphasize relatively simple procedures like the Ames test.

In attempting to carry on toxicological research, federal civil servants face the chronic problems of stop-and-go projects and stop-and-go financing. Complicating matters are the ideological attitudes of some of the key leaders having voice in toxicological matters. There is a definite anti-industry bias and an inclination to exaggerate the hazards posed by chemicals. An attitude has arisen that affects some people from academia as well as those in government. It is that the tiniest, poorly based suspicion of toxicity of a chemical demands instant widespread publicity and drastic federal action. Under such circumstances scientific evidence and truth do not fare very well. In such an atmosphere it is highly unlikely that competent testing or research can be conducted.

In the light of these comments about toxicological activities elsewhere, it should be evident that this Institute can play an extremely important role. It can establish high standards of excellence and truth. If this is done, the excellence will be slowly but surely broadly recognized and sloppy work and sloppy interpretations by others will be discredited.

I come now to a broader major topic—the public relations problem that chemical industry is currently experiencing. The problem has many causes and facets. At the root is public ignorance about science and specifically about chemistry. People tend to be afraid of the unknown and of what they cannot comprehend or assess. People tend to believe that nameless conspirators are seeking to harm them. The public's fears are played on by careless journalists and by opportunists. And so there is confusion about a so-called cancer epidemic. Higginson's earlier remarks that 80–90% of cancer is environmentally related is misinterpreted to signify that industrial chemicals represent a great cancer hazard. The fact is that Higginson and other reputable epidemiologists believe that only 1–6% of cancer is occupationally related.

The federal government does not go quite as far as some individuals in its tactics. But the recent draft of the report of the Toxic Substances Strategy Committee (TSSC) prepared for President Carter goes pretty far. The report seems to have as a principal objective a vast expansion in government regulatory appropriations and activities aimed at the chemical industry.

Typical of the approach of the report is the following, and I quote: "Perhaps the most serious source of human exposure to toxic chemicals is the workplace. More than 100,000 workers are believed to die each year as a result of physical and chemical hazards at work..." Note that after emphasizing the words toxic chemicals the report proceeds to lump in physical with chemical effects. That is, every time a construction worker falls off a scaffold, chemicals are largely to blame.

The report seems designed to create panicky fears about the dangers of chemicals. Since large scale morbidity cannot be demonstrated now the report emphasizes potential delayed effects.

One of the questionable aspects of the TSSC report is that it quotes a discredited document that was released on 15 September 1978 by staff members at the National Cancer Institute, the National Institute of Environmental Health Sciences, and the National Institute for Environmental Safety and Health. The report was entitled "Estimates of the Fraction of Cancer in the United States Related to Occupational Factors". The document advanced the claim that occupational exposure to carcinogens is a factor in an estimated 20–38% of all cases of cancer. The context of the claim is such as to put the blame on chemicals in the workplace. Examination of the original document shows it to be indefensible. Had it been subjected to peer review it would have been cut to pieces. After its release it was roundly criticized by Cuyler Hammond and others, including Richard Doll, Regius Professor of Medicine at Oxford. He categorized the report as "scientific nonsense". However, its effects linger on and are perpetuated in a document prepared for President Carter. The British medical journal the *Lancet*, pointed to numerous defects in the report and concluded with: "It is sad to see such a fragile report under such distinguished names".

A major component in that estimate of chemical carcinogens is the purported cancer mortality due to asbestos. Asbestos accounts for 13–18 of the 20–38%, that is, a half or more. We all know that asbestos is a mixture of chemicals but it is obtained by mining and not from the chemical industry.

There is no doubt that very heavy exposure to asbestos combined with cigarette smoking leads to high cancer mortality. However, heavy exposure tends to be limited to a small group of asbestos workers. The large estimated number of projected asbestos-related cancer fatalities was arrived at by inflated guesses that 11,000,000 workers had been substantially exposed. These include 4,500,000 World War II shipyard workers. I can testify from personal experience that at the Philadelphia Navy Yard, asbestos exposure involved only a tiny fraction of personnel. Moreover, because of the war-time military draft, few young people were employed. The average age of workers was 40–45. That was 35 years ago. Whatever contribution those workers were going to make to cancer statistics has already been largely made.

I now wish to discuss a problem that will constitute a running sore for the chemical industry unless it is treated promptly and responsibly. I refer to the matter of the chemical waste dumps. No stream of excuses or attempts to blame others for the troubles will suffice. Moreover, the public does not differentiate well between companies. If one company has

caused a problem, all companies share in the blame. Cleaning up the situation will be expensive but if it is done responsibly and intelligently, the costs are likely to be far less than those that will be incurred financially and indirectly if thoughtful action is not taken.

To approach the matter of handling the waste dumps requires a combination of many branches of science including hydrology, geochemistry, physical chemistry, microbiology and chemical engineering as well as toxicology. I suggest that the matter might be tackled by a task force created by a consortium of companies. It might well be desirable to create an organization similar in form and financing to the Chemical Industry Institute of Toxicology to provide the necessary expertise and research capability to handle waste-disposal problems.

By training and experience chemists and chemical engineers are great problem solvers. They know how to meet needs of society. They have long experience in bringing on new products and phasing out old ones. They are accustomed to changing with the times. I have talked to many leaders of chemical industry and they have unanimously expressed a policy of acting responsibly and in the public interest. I have been impressed with their sincerity. In general, more than one chemical product will serve a specific need. If problems of toxicity arise with one product, another can and will be substituted. I believe the most the chemical industry can hope for is that facts regarding hazards will be established and that truth will prevail. By a steadfast search for truth and the fearless dissemination of it, this Institute can render great service to the industry and to society. By establishing this Institute and providing for its support and integrity, the chemical industry has taken positive action. Implementation of this trust now passes into the hands of the scientific and technical staff.

All of us here, today, wish them well.

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REVIEWS OF RECENT PUBLICATIONS

Asbestos. Vol. 1. Properties, Applications, and Hazards. Edited by L. Michaels and S. S. Chissick. J. Wiley & Sons, Chichester, 1979. pp. xi + 553. £25.00

This book presents an extremely comprehensive and well-balanced exposition of the use of asbestos, the monitoring and identification of asbestos fibres, exposure to asbestos, and asbestos-related diseases. Each chapter is contributed by an expert on the subject. These experts range from mineralogists and physicists to pathologists and epidemiologists.

The geological occurrence, types and structure of the asbestos minerals are dealt with in two early chapters. Their fibrous character, tensile strength and resistance to high temperatures, which make them so important commercially, are discussed and particular attention is paid to their different surface characteristics. The fact that the surface properties of asbestos can be altered in various ways is mentioned, although the very recent data indicating that metal leaching of the fibre may occur in biological fluids are omitted.

The attitudes to asbestos shown by different groups such as governments, employers, trade unions and the general public are lucidly discussed, and so are the occupational and non-occupational exposure of man to asbestos and the redistribution of asbestos in the environment. One chapter is devoted to the monitoring and identification of airborne asbestos, another to its identification in solid materials. There is an extremely thorough chapter dealing with regulations on the use of asbestos and with its control in a working environment. This latter topic is also discussed by several other contributors.

Before asbestos-related diseases are considered in detail, it is made clear that current substitutes are often technically inferior to asbestos and that there are limited data on the risks associated with some of the substitutes.

Five chapters are concerned with asbestos-related diseases. These deal with the pathological changes associated with the inhalation of asbestos—asbestosis, carcinoma, mesothelioma and pleural plaques. The clinical features of each condition are clearly described, as well as the macro- and microscopic appearances at post-mortem examination. Experimental pathology is discussed, but not the difficulties of extrapolating the results of animal experiments to man, and there is a lucid discussion on the epidemiology of asbestos-related diseases.

The final chapter in this volume concerns methods of preventing asbestos-related diseases, including the physical control of human exposure to asbestos and medical management. In it are also discussed synergistic co-factors such as cigarette smoking, prior occupational exposure to inhaled carcinogens, immunodeficiency and individual susceptibility. This book is marked Volume 1 and we look forward with great interest to the appearance of a companion volume.

Environmental Carcinogens—Selected Methods of Analysis. Vol. 2—Methods for the Measurement of Vinyl Chloride in Poly(vinyl chloride), Air, Water and Foodstuffs. By D. C. M. Squirell and W. Thain. IARC Scientific Publications no. 22. International Agency for Research on Cancer, Lyon, 1978. pp. xiii + 142. Sw. fr. 75.00 (available in the UK through HMSO).

This volume continues the series that was begun with methods of analysis for nitrosamines (*Cited in F.C.T.* 1979, **17**, 535) and is equally well presented. An introductory chapter by L. Griciute deals with the carcinogenicity of vinyl chloride (VC) and with legislation designed to limit exposure to the monomer. The named authors then provide a general review of approaches to the monitoring and measurement of VC. Ionization and spectroscopic detection methods are discussed, as well as those based on chemical, thermal and electrical properties, and separation techniques (mainly involving gas chromatography) are mentioned. Sampling techniques for specific applications are covered, and there are sections on the detection of VC in polymers, food and food simulants, and on the preparation of VC standards. Unfortunately, few recommendations are made about which methods are best and the number of references to 'personal communications'—41, compared with 101 listed references—seems disproportionate.

The section on methods of analysis contains eight carefully written procedures, five dealing with VC analysis in air, and one each with its detection in aqueous liquids, foodstuffs and polyvinyl chloride. Each method is preceded by a brief description of its scope and application, the principle on which it is based and any potential hazards. As in the first volume, the recommended ISO format is used to set out the methods. Head-space gas-chromatographic analysis is used in three of the procedures; such analysis is being considered by ISO as a basis for an international standard.

This section is followed by methods for the preparation of calibration standards of VC in nitrogen or air, water and organic solvents. These are presented in the same clear style that characterizes the rest of this useful publication.

Report of the Government Chemist 1978. Department of Industry: Laboratory of the Government Chemist. HMSO, London, 1979. pp. 170. £4.25

One of the tasks of the Laboratory of the Government Chemist over the years has been the analysis of wildlife and foodstuffs for pesticide residues. In 1978, samples of birds of prey still contained residues of dieldrin, despite the 1974 ban on its use as a cereal-seed dressing, as well as *p,p'*-DDE and PCB. However, in 100 samples of breast meat from wood

pigeons shot in East Anglia at the time of spring-wheat sowing, levels of dieldrin were below 0·01 mg/kg in over 80% and below 0·03% mg/kg in most of the remainder. No carbophenothion or chlorfenvinphos, now increasingly used as alternatives to dieldrin, could be detected. Of 34 samples of milk from farms where sewage sludge had been used as a fertilizer, only one contained an enhanced level of dieldrin, and this came from an area where the pesticide is used industrially for moth-proofing.

Although mercury levels in fish are not regarded as a general problem, there has been some concern that populations consuming large amounts of fish might be at risk. A survey of fishing communities of the northern Irish Sea- and south-west-coasts in 1978 revealed that mercury levels in fish were significantly higher in the former area. However, blood levels of mercury in subjects with the highest intakes were still at least an order of magnitude below the toxic threshold estimated by WHO. We recently published an abstract of the detailed report of this study (*Cited in F.C.T.* 1980, **18**, 203).

During 1978 much attention was given to the analysis of substances in water. These included polycyclic aromatic hydrocarbons, for which a high-performance liquid chromatographic method of separation has been developed. A start was made on examining levels of chlorite formed in water treated with chloride dioxide. The results of this study should assist the Department of the Environment in assessing any potential health hazard from such treatment. The laboratory has also been involved in the analysis of low levels of halogenated hydrocarbons in water. In a routine determination of arsenic and selenium, over 1000 samples of drinking water were analysed. None had an arsenic content greater than the WHO recommended limit of 50 μg/litre, and only two exceeded 10 μg/litre. Similarly, the highest selenium level of 5·9 μg/litre was well below the WHO and EEC proposed limit of 10 μg/litre, and 92% of samples contained less than 1 μg/litre.

Effort devoted to the development of methods for analysis of toxic substances in air increased during the year. A principal source of attention was a continuous personal monitor for toluene diisocyanate (TDI), designed to be worn by a worker throughout an eight-hour shift. This was found to measure TDI levels to a precision of about 15%. Work also started on a new test for benzene on behalf of the HSE, to meet the possible reduction of the TLV to 1 ppm by volume.

Smoke from six brands of cigarettes made with flue-cured tobacco and four brands of cigarettes made with air-cured tobacco was analysed for volatile nitrosamines. *N*-Nitrosodimethylamine was found in smoke from all the cigarettes, except from two flue-cured brands. *N*-nitrosodiethylamine, 1-nitrosopyrrolidine and 1-nitrosopiperidine were also found in isolated cases. A high nitrate/nitrite level in the tobacco appeared to be associated with high nitrosamine levels in the smoke, but no correlation was evident between nitrosamine levels and tar or nicotine levels.

The Handbook of Cancer Immunology. Volumes 1–4. Edited by H. Waters. Garland STPM Press, New York, 1979. pp. viii + 344, viii + 276, viii + 434 & viii + 335, respectively. $37.50 each.

The aim of this series is to provide researchers, clinicians and administrators with a summary of current research and knowledge in this field. The first volume has a number of comprehensive chapters covering the phylogeny and development of basic immunological functions. These include both the cellular mechanisms associated with the induction and regulation of immune reactions and the different humoral immune responses. Particular attention is paid to host–tumour relationships and to the recent observations that dissimilar cell surface-membrane antigens present on tumour cells are capable of evoking different host responses. Two interesting concepts are discussed at length, namely the way genetic factors may influence the nature and intensity of immune responses against tumour-associated neoantigens, and the organ specificity of many immune defences against tumours.

The latter concept, dealt with by Dr. A. E. Reif, is of particular interest, as it suggests that various organs possess individual as well as systemic defences, and demonstrates that the effects of a carcinogen on the target organ generally outweigh the systemic effects for many types of oncogenesis. According to this author, any substance that damages the specific defences of an organ that is already the target of industrial or environmental carcinogens would be a most effective co-carcinogen.

The second volume details the biological mechanisms that allow some tumours to escape immune destruction. One of the major areas covered is that of antigenic modulation and induction of tolerance. The various means of acquiring tolerance, including the ability of antigen–antibody complexes to render lymphocytes insensitive to activation signals, are discussed in depth. With regard to tumour immunity, the factors affecting tolerance induction appear to be the age of the host at the time of initial encounter with the antigen, the antigen dose and its molecular nature. Other tumour escape mechanisms are also discussed, and it appears that further knowledge of these mechanisms would provide insight into ways to reverse such escape, the real aim of immunotherapy.

In volumes 3 and 4 several contributors attempt to relate immune status to the treatment and prognosis of various forms of cancer. Whilst it is clear that longitudinal studies using improved immunological techniques are required for more definitive analysis of complex immune defences against human cancer, these chapters contain much important and recent information. Amongst the topics discussed are the delayed cutaneous hypersensitivity reactions to solubilized tumour-cell extracts, serological diagnostic techniques for studying humoral immune reactions, and the role of cells of the mononuclear phagocytic system. There now appears to be strong suggestive evidence that impairment of macrophage function may be a factor in tumour growth.

This handbook thus contains much relevant and important up-to-date information and should interest everyone concerned with interrelating immunology with the biology and growth of cancer cells.

A History of Microtechnique. The Evolution of the Microtome and the Development of Tissue Preparation. By B. Bracegirdle. Heinemann, London, 1978. pp. xiv + 359. £22.50.

It would be wrong to dismiss this book as another literature survey, although at first glance that is what it appears to be. Painstakingly researched and stylishly written, it provides an entertaining look at the origins and development of microtomy and specimen preparation up to 1910.

A historical survey such as this may appear to have little to offer the modern histologist, but this is not true. An awareness of the techniques in use at the time leads to an appreciation of the skill and patience of those responsible for the superb preparations of the day. A histologist's life is so much easier nowadays, with reliable stains, sophisticated embedding and sectioning equipment and the like, that it is easy to forget the primitive beginnings of the histologist's art. Describing, as it does in great detail, the practical aspects of histological technique, this book may assist in redirecting the bench workers thoughts toward the niceties of technique and away from the rather inflexible scientific approach.

Therein lies the usefulness of this well-prepared text. Superficially it is a comprehensive survey of historical material. Hidden beneath this surface veneer is a tribute to all the pioneers of histological technique who, by trial and error, laid the foundation stones of modern histology.

As a reference book this work is outstanding. As value for money it is, regrettably, questionable. The text is well furnished with line drawings but the 53 plate illustrations are disappointing. In a book of this quality and cost, the reader is entitled to more than four colour plates—particularly when biological material is depicted, as this loses much of its impact in monochrome. It is unfortunate that the high price is likely to deter all but the most affluent from possessing an excellent and enjoyable work of reference.

Statistical Treatment of Experimental Data. Physical Sciences Data 2. By J. R. Green and D. Margerison. Elsevier/North-Holland Biomedical Press, Amsterdam, 1978. pp. x + 382. Dfl. 90.00.

This is a clear well-written book covering a wide range of elementary statistical techniques. Unlike many elementary texts, the ideas and reasoning behind the statistical methodology are presented most satisfactorily. In addition to providing a basic grounding in statistical inference, the book gives the reader a firm grasp of the theoretical concepts, an essential if the assumptions that limit the use of the various techniques are to be understood.

A significant proportion of the text is devoted to the development of probability theory and probability distributions. An understanding of these topics is vital if the use of statistical analysis is not to be abused. The book also has chapters on hypothesis testing, goodness of fit tests, analysis of variance and correlation as well as some good chapters on regression. The authors also provide, where appropriate, general considerations on methods of data acquisition, the planning of experiments and the extent to which these concepts relate to the choice of a particular statistical method.

This text is readable as well as useful and should bring the reader to a point from which he can proceed to analyse his data with the confidence that follows from an understanding of the problems posed by data analysis and its limitations.

GLC and HPLC Determination of Therapeutic Agents. Part. 3. Edited by K. Tsuji. Marcel Dekker, Inc., New York, 1979. pp. xiv + 548. Sw.fr. 106.00.

The last issue (*Fd Cosmet. Toxicol.* 1980, **18**, 194) carried a review of Parts 1 and 2 of this three-part work on the chromatographic analysis of therapeutic agents. The final part has now appeared, continuing the detailed consideration of the analysis of specific classes of compound begun in Part 2.

This latest volume greatly enhances the value of the series. The broad classes of substances it covers include the prostaglandins, nucleosides and nucleotides, alkaloids, antihistamines, vitamins, amino acids and peptides, sugars and lipids.

Finally author and subject indexes covering all three volumes are provided.

BOOKS RECEIVED FOR REVIEW

Cancer Registration and its Techniques. Edited by R. MacLennan, C. Muir, R. Steinitz and A. Winkler. IARC Scientific Publications no. 21. International Agency for Research on Cancer, Lyon, 1978. pp. xii + 235. Sw.fr. 40.00 (available in the UK through HMSO).

Safe Use of Pesticides. Third Report of the WHO Expert Committee on Vector Biology and Control. World Health Organization Technical Report Series No. 634. WHO, Geneva, 1979. pp. 44. Sw.fr. 5.00 (available in the UK through HMSO).

Banbury Report. 1. Assessing Chemical Mutagens: The Risk to Humans. Edited by V. K. McElheny and S. Abrahamson. Cold Spring Harbor Laboratory, New York, 1979. pp. xiii + 367. $38.00.

Recent Results in Cancer Research 66. Carcinogenic Hormones. Edited by C. H. Lingeman. Springer-Verlag, Berlin, 1979. pp. 196. $42.90.

Cadmium Toxicity. Edited by J. H. Mennear. Marcel Dekker, Inc., New York, 1979. pp. viii + 224. Sw.fr. 58.00.

Environmental Health Impact Assessment. Report on a WHO Seminar, Argostoli, Kefalonia, Greece, 2–6 October 1978. EURO Reports and Studies 7. Regional Office for Europe, World Health Organization, Copenhagen, 1979. pp. 31. Sw.fr. 5.00 (available in the UK through HMSO).

Nutritional and Safety Aspects of Food Processing. Edited by S. R. Tannenbaum. Marcel Dekker, Inc., New York, 1979. pp. x + 448. Sw.fr. 100.00.

Oncogenesis and Herpesviruses III. Part 1: DNA of Herpesviruses, Viral Antigens, Cell-Virus Interaction. Edited by G. de-Thé, W. Henle and F. Rapp. IARC Publications no. 24. pp. lv + 580. Sw.fr. 50.00.

Carcinogenic Risks: Strategies for Intervention. Proceedings of a Symposium organized by IARC and the French National Institute of Health and Medical Research, held at the International Agency for Research on Cancer, Lyon, France, 30 November–2 December 1977. Edited by W. Davis and C. Rosenfeld. IARC Publications no. 25. pp. xix + 283. Sw.fr. 50.00.

Handbook of U.S. Colorants for Foods, Drugs, and Cosmetics. By D. M. Marmion. John Wiley & Sons Ltd, Chichester, 1979. pp. vii + 350. £13.25.

Environmental Health Criteria. 11. Mycotoxins. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 127. Sw.fr. 11.00 (available in the UK through HMSO).

Progress in Experimental Tumor Research. The Syrian Hamster in Toxicology and Carcinogenesis Research. Edited by F. Homburger. S. Karger AG, Basel, 1979. pp. x + 442. Sw.fr. 163.00.

Mutagenesis in Sub-Mammalian Systems. Status and Significance. Edited by G. E. Paget. MTP Press Ltd., Lancaster, 1979. pp. xiv + 231. £8.95.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 71. Edited by F. A. Gunther. Springer-Verlag, New York, 1979. pp. viii + 181. $24.20.

Industrial and Environmental Xenobiotics. *In vitro* Versus *in vivo* Biotransformation and Toxicity. Proceedings of an International Conference held in Prague, Czechoslovakia, 13–15 September 1977. Edited by J. R. Fouts and I. Gut. Excerpta Medica, Amsterdam, 1978. pp. xvii + 345. Dfl. 130.00.

Handbook of Clinical Neurology. Vol. 37. Intoxications of the Nervous System. Part II. Edited by P. J. Vinken and G. W. Bruyn. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. xii + 578. Dfl. 245.00.

Drug Metabolism in Man. Edited by J. W. Gorrod and A. H. Beckett. Taylor & Francis Ltd., London, 1978. pp. xii + 267. £12.00.

Hartley's Microscopy. By W. G. Hartley. Senecio Publishing Co. Ltd., Oxford, 1979. pp. x + 220. £9.75.

Dangerous Chemical Substances and Proposals Concerning their Labelling. 4th Ed. Council of Europe. Maisonneuve éditeur, Moulins-lès-Metz, France, 1978. pp. 956. F.fr. 330.

Residue Reviews. Vol. 72. Residues of Pesticides and Other Contaminants in the Total Environment. Edited by F. A. Gunther. Springer-Verlag, New York, 1979. pp. viii + 154. $18.00.

Asbestos Killer Dust. A Worker/Community Guide: How to Fight to Hazards of Asbestos and its Substitutes. By A. J. P. Dalton. BSSRS Publications Ltd., London, 1979. pp. 287. £2.25.

Drug Toxicity. Edited by J. W. Gorrod. Taylor & Francis Ltd., London, 1979. pp. viii + 326. £12.50.

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Information Section

ARTICLES OF GENERAL INTEREST

MORE CHAPTERS IN THE BRACKEN SAGA

Bracken (*Pteridium aquilinum*) has been implicated in the induction of tumours in cattle (*Cited in F.C.T.* 1968, **6,** 108; *ibid* 1970, **8,** 85). Some workers have attributed its carcinogenicity to shikimic acid, which induces tumours of the gastric mucosa when fed to mice (*ibid* 1975, **13,** 405), but the feeding of shikimic acid to rats has failed to induce tumours (*ibid* 1978, **16,** 630). Other carcinogenic principles have been suggested, including a more potent compound, pterosin B and its chemical relatives among the sesquiterpenes (*ibid* 1976, **14,** 514), some of which appear capable of inducing tumours in the alimentary tract and the bladder of cattle and experimental animals.

Yoshihira *et al.* (*Chem. pharm. Bull., Tokyo* 1978, **26,** 2346) have described the fractionation of organic-solvent extracts of young bracken fronds (*P. aquilinum* var. *latiusculum*) and the characterization of their constituents. Successive partitioning of a methanolic extract of young bracken fronds with hexane, benzene, ethyl acetate and butanol demonstrated more than 20 sesquiterpenes with the 1-indanone nucleus (pterosins) and their glycosides (pterosides), together with benzoic, *p*-hydroxybenzoic, vanillic and *p*-coumaroylquinic acids, vanillin, kaempferol and the steroids β-sitosterol, stigmast-4-en-3-one and 5-α-stigmastane-3,6-dione. A later paper by the same team (Fukuoka *et al. ibid* 1978, **26,** 2365) describes the chemical and physical characterization of 23 sesquiterpenes derived from young bracken fronds. Cytotoxicity testing against HeLa cells, acute sc and oral toxicity tests in rats, mice and guinea-pigs, and feeding tests in rats were carried out on various fractions and on the dried fronds or rhizomes of the plant (Yoshihira *et al. loc. cit.*). Several fractions showed cytotoxicity for HeLa cells. Further separation of these led to the isolation of very small amounts of a number of indanone derivatives as well as several phenolic compounds and steroids. Some of the indanone compounds were cytotoxic and all of these had a similar effect on the HeLa cells, which were reduced in size, showed a tendency to become spindle-shaped and were characterized by a clear, sometimes vacuolated, cytoplasm, a rounded nucleolus, and a decrease in mitotic figures. There was, however, no indication of the occurrence of any alkylation. A diet containing 30% dried bracken rhizome was fed to guinea-pigs for 5 days, and residues from extractions as well as dried samples of bracken frond and rhizome were fed to rats for 35–205 days again at levels of 30% in the diet. In addition, various fractions were mixed with the diet in ratios calculated to provide levels 1·6–3 times as high as did the dried-bracken diets. Ileal adenoma and papillary adenocarcinoma occurred in rats fed the dried frond or rhizome, but diets containing pterosin B and pteroside B failed to induce any significant incidence of tumours, and thus provided no support for their involvement in bracken carcinogenicity.

The approximate amounts of these two compounds administered in the diet were tabulated as 4 and 10 mg/day, respectively, in the report of the study (Yoshihira *et al. loc. cit.*) but were quoted as 4 and 10 mg/kg/day (for 120 days) in another paper by this group (Fukuoka *et al. J. Pharmacobio-Dyn.* 1978, **1,** 324). The latter paper reports the mutagenicity testing of the bracken-derived compounds and extracts in *Salmonella typhimurium*, strains TA100 and TA98. All 15 pterosins tested gave negative results in concentrations up to 1 mg/plate. On the other hand, kaempferol, one of the flavonoids isolated from bracken, showed marked mutagenicity in both strains of *S. typhimurium* when tested in the presence of S–9 mix, but not in its absence. Since it was known that the glucosides of pterosin-sesquiterpenoids are hydrolysed before being absorbed from the intestine of rats, some of the bracken extracts were pretreated with 'hesperidinase', as a model for the glycosidases of the intestinal flora, before being subjected to the Ames test. Using these procedures, the mutagenic activity of the boiling-water extract of bracken was traced to a fraction consisting principally of astragalin (kaempferol 3-β-D-glucoside). This was only active, however, after the 'hesperidinase' treatment, which hydrolysed astragalin to kaempferol. Another compound, which was more water-soluble than astragalin, was also shown to have some mutagenic activity. The authors of this paper are extending their work on the mutagenic potential of flavonoids, which are common components of naturally-occurring materials. The group is also continuing its studies of the bracken sesquiterpenes, and earlier this year reported the characterization of more than 30 1-indanone derivatives from bracken and the demonstration that the sesquiterpene constituents of the rhizomes differ from those of the fronds, particularly in the relative amounts of glucosides and the stereochemistry of several compounds at the C-2 and C-3 positons (Kuroyanagi *et al. Chem. pharm. Bull., Tokyo* 1979, **27,** 592). The rhizomes of bracken have been shown to be more carcinogenic to rats and more toxic to cattle than are the young fronds (*Cited in F.C.T.* 1974, **12,** 285), but whether this is a reflection of any differences in their sesquiterpene chemistry has yet to be established.

The findings of Hirono *et al.* (*Gann* 1978, **69,** 383), who compared, in small groups of rats, the carcinogenicity of aqueous extracts of bracken with that of dried fronds, provided further evidence for the solubility of a bracken carcinogen in boiling water. One

group of animals (group I) received a diet containing 33% dried bracken powder for 3 months. A second (II) received for 16 months as drinking-water an infusion prepared from 60 g dried bracken in 2 litres boiling water. A third (III) received a 72-hr cold-water infusion of bracken (60 g/2 litres) for 16 months, while a fourth (IV) was fed for up to 12 months a basal diet containing, at a level of 33%, a boiling-water extract of bracken that had been concentrated by evaporation to a tarry liquid. In a second experiment, rats received a basal diet containing a concentrated triple hot-water extract of bracken for up to 16 months. Surviving animals in all these groups were killed 480–500 days after the start of the treatment. In group I, 13 of the 14 rats, all of which survived for longer than 10 months, developed multiple ileal tumours, comprising adenomas and adenocarcinomas, and one also had a bladder tumour. In group II, six of the ten rats surviving longer than 7 months developed ileal or bladder tumours or both. There were no tumours in group III, but in group IV and in the treated rats in the second experiment, all but one rat had bladder tumours and most also had ileal tumours. These results suggest that the carcinogen present in bracken is water-soluble and extractable by boiling water, but is not appreciably leached out by cold water.

A somewhat different approach to the question of bracken-induced carcinoma has been put forward by Jarrett *et al.* (*Nature, Lond.* 1978, **274,** 215). Virus-induced papillomas of the alimentary tract are common in British cattle and occur in animals raised on farms with and without areas of bracken. Cattle with alimentary carcinomas show a much more restricted geographical distribution than those with papillomas, but it has been noted that in the areas of high cancer incidence, the papillomas occur in greater numbers in individual animals and are found at multiple sites. In the bracken-infested upland areas of Scotland and the north of England there is a high incidence of alimentary cancer in beef cows, in contrast to the neighbouring, relatively bracken-free, lowland areas. In a detailed study of these cattle Jarrett *et al.* (*loc. cit.*) found that papillomas and carcinomas occurred at the same sites, and in some cases there was clear evidence of the progression of the one to the other. On the basis of this work and of epidemiological studies reported elsewhere, they suggest that the long-term effect of consuming bracken at levels too low to cause the radiomimetic lesions of acute bracken poisoning may promote persistent papillomatosis and progression of the papillomas to malignant tumours, possibly by inhibiting normal immunological reactions to the papilloma virus.

[P. Cooper—BIBRA]

ALCOHOL AND PREGNANCY—PART 1: ANIMAL DATA

Early this year the US Department of the Treasury launched an educational programme to warn the public of the dangers of drinking during pregnancy. However a clear picture of the various effects of different levels of drinking has not yet emerged from the considerable animal and human data on this topic (*Cited in F.C.T.* 1978, **16,** 290). We now report the results of some more recent studies in animals.

The effects of chronic and acute maternal ethanol intake on foetal development were compared by Henderson *et al.* (*Alcoholism clin. expl Res.* 1979, **3,** 99). Groups of 15 female rats were given a liquid diet containing 6% ethanol or its isocalorific equivalent for at least 30 days before mating and for 20 days of gestation. The acute exposure groups, of 11–13 dams, were given two doses/day of 5 g/kg ethanol by gastric intubation on days 11–13 or 14–16 of gestation. The acute dose was selected to give roughly the same 24-hr intake as in the chronic-exposure group. Blood levels were about 70–200 mg/100 ml in chronic-exposure groups and reached peaks of 100–300 mg/100 ml in acute-exposure groups. Resorption rates in all the ethanol-exposed rats were significantly higher than for controls and highest in groups exposed chronically or acutely on days 11–13. There was also an increase in the number of dead foetuses among animals exposed chronically. Body weights of all the treated groups were 20–25% less than those of the controls and organ weights were also reduced in the treated animals although there were few significant differences when relative organ weights were compared. Tissue protein tended to be higher in the groups exposed to ethanol. Both acute and chronic exposure had adverse effects on foetal viability and growth.

In a study using mice, Randall & Taylor (*Teratology* 1979, **19,** 305) investigated the effects of different doses of alcohol. They administered the ethanol in a liquid diet at doses providing 17, 25 or 30% of the total calories as ethanol from days 5 to 10 of gestation. The dams were killed and the foetuses were examined on day 19. Results are given for groups of 14 to 21 dams. The number of implantation sites was significantly greater in the group given the diet providing 25% of the calories as ethanol than in the controls. There were significantly more resorptions and malformations in the two groups given the highest doses but not in the low-dose group. The malformations produced were of a wide variety of types. Blood alcohol levels were negligible in the low-dose group, 36–261 μg/100 ml in the mid-dose group and 124–384 mg/100 ml for the high-dose group.

Yanai & Ginsburg (*Alcoholism clin. expl Res.* 1977, **1,** 325) investigated the effects of chronic ethanol exposure in two species of mice. One group was given 10% ethanol in water sweetened with dextrose and saccharin as its sole liquid supply (the ethanol provided up to 20% of the total calories), while control groups were given sweetened water or tap water *ad lib.* until 60 days of age. At this age they were mated to animals of the same strain (C57 or DBA) given the same pretreatment. Pregnant females continued to receive ethanol or control drinking water until day 14 after parturition. Their progeny were never directly

exposed to ethanol. The ethanol intake of the parents was 10–15 g/kg body weight/day at the time of mating and 12–38 g/kg/day by nursing females; blood ethanol levels rose at night to 20–45 mg/100 ml, falling to zero during the day. No significant difference between the two strains was noted in these respects. The size and survival of litters born to parents given ethanol were normal. The C57 pups reached normal body weight during the first 28 days of life, whereas an initially lower weight in treated DBA pups at 5–15 days was corrected by 28 days. The ages at which hair appeared and at which the startle and righting responses developed were similar in treated and control groups. In DBA but not C57 pups of ethanol-exposed mice, the eyes opened 1 day later than in controls; DBA pups in the exposed group also showed some delay in ear development. The offspring of animals given ethanol showed normal reproductive abilities.

Schwetz *et al.* (*Teratology* 1978, **18,** 385) gave 15% ethanol in drinking water to pregnant mice and rats on days 6–15 of gestation, and to pregnant rabbits on days 6–18, corresponding with the periods of major organogenesis in these species. Blood ethanol levels reached 200 mg/100 ml in mice and 28–40 mg/100 ml in rats and rabbits. The animals were killed just before normal delivery. All treated animals showed reduced fluid intake and decreased maternal body weight. In rats and mice the number of live foetuses per litter was not significantly affected but foetal weight and length were significantly decreased by comparison with control values. In rabbits there was an increase in the number of resorptions but this was mainly due to the complete resorption of two litters. In no species was there any increase in the incidence of external or soft-tissue malformations. There were more minor skeletal abnormalities in mice and rats, more probably due to retardation of foetal growth than to specific ethanol toxicity. There were no unequivocal teratogenic reactions. In a second study (Øisund *et al. Acta pharmac. tox.* 1978, **43,** 145) female rats were given 12% ethanol in their drinking water to provide 20–25% of their total calories. One group was given the treatment for 3–4 wk before and during pregnancy, a second was treated during suckling only and a third group was treated before and during pregnancy and during suckling. Blood ethanol levels reached 33–75 mg/100 ml. Litter size was significantly reduced in the latter group by comparison with animals given isocalorific amounts of soya oil in place of ethanol, but no other change in reproductive performance was observed. The pups were examined for up to 24 days and showed in some instances increased body weight and increased weight of brain, liver, kidney and heart muscle at days 2–14 of life, but no overall tendency to higher organ weight over days 0–24. Only two out of 321 pups of ethanol-treated dams had gross malformations (one hindquarter bony abnormality and one hydrocephalus) compared with none out of 444 controls. There was thus no evidence of significant teratogenicity.

A number of workers have concentrated on more specific aspects of the foetal effects of ethanol. Buckalew (*Res. Commun. Psychol. Psychiat. Behav.* 1978, **3,** 353) reported behavioural differences in pups of albino mice given 5% ethanol in their drinking water during nursing. (There was no prenatal exposure.) The spontaneous activity of exposed pups was significantly decreased by comparison with that of controls (from a different litter of the same parents). The behavioural effect was attributed to a pharmacological reaction to ethanol, rather than to a neurological defect.

A neurotoxic function of ethanol is suggested by the results of an experiment by Khawaja *et al.* (*Res. Commun. chem. Path. Pharmac.* 1978, **22,** 573). They found that in rats the ingestion of 9% ethanol or 9% 1,3-butanediol as their liquid source during pregnancy and lactation led to marked inhibition of neuronal protein synthesis in pups at 18 days of age. In contrast the same exposure stimulated amino acid incorporation by neuronal perikarya when the pups were 8 days old. The *in vitro* synthesis of liver protein was enhanced both at 8 and 18 days in liver slices obtained from pups of exposed parents, and this was attributed to increased amino-acylation of transfer RNA in the pH 5 fraction of liver enzymes.

Beskid *et al.* (*Expl Path.* 1978, **15,** 355) gave 40% ethanol in doses of 8 g/kg/day by gastric tube to mice from 3–4 days before impregnation to the end of gestation, and studied liver samples taken from the pups 2–8 hr after birth. The characteristic changes observed were lipid accumulation, a strong acid-phosphatase reaction, and reduced succinic dehydrogenase activity. There was no difference in alcohol dehydrogenase activity in livers from exposed and control animals. Ultrastructural changes observed in livers of exposed animals included depletion of rough endoplasmic reticulum and swelling and distortion of some mitochondria. Prasad *et al.* (*Obstet. Gynec., N.Y.* 1978, **52,** 318) found that lactic dehydrogenase activity was diminished by ethanol exposure in the mouse ovary but not in the testis. Lactic dehydrogenase was measured 90 days after delivery in the ovaries of both mothers and female offspring and in the testicular tissues of male pups, after ip injections into the mothers of 100 µl 70% ethanol/day on days 9–16 of gestation.

A detailed investigation of the effects of ethanol on brain development was made by Bauer-Moffett & Altman (*Brain Res.* 1977, **119,** 249). Infant rats were exposed directly to 3·4–4·0% ethanol by inhalation during days 3–20 of life. Maximal blood ethanol levels produced by this method of administration averaged 239 mg/100 ml. Brain but not body growth was significantly stunted, and growth of the cerebellum was even more seriously affected. The effects of ethanol on brain growth were seen shortly after the start of exposure and persisted in the adult animal. Ethanol diminished growth in the anterior and posterior lobes and in all layers of the cerebellar vermis, but its effect was most pronounced in the anterior lobe, particularly in its medullary layer. Light microscopy (Bauer-Moffett & Altman, *loc. cit.*) showed that the numbers of Purkinje's cells in the vermal lobules were reduced after 2 days of ethanol inhalation, and that this change could not subsequently be reversed either by further ethanol exposure or by post-weaning rehabilitation. Autoradiography showed that granule-cell neurogenesis in the rat cerebellum was not significantly altered by exposure to ethanol, and it appears that the initial targets of ethanol are the immature Purkinje's cells.

Since ethanol is rapidly metabolized in the body, O'Shea & Kaufman (*J. Anat.* 1979, **128**, 65) investigated the possible teratogenic properties of its primary metabolite acetaldehyde. To study these effects free from the effects of ethanol they injected mice with acetaldehyde during the early post-implantation period when critical morphogenetic events occur. Groups of 7–11 pregnant female mice were injected iv with 0·1 ml 0·9% saline/25 g body weight or a similar volume of saline containing 1 or 2% (v/v) acetaldehyde on days 7, 8 and 9 of gestation and were autopsied on days 10 or 19. The 2% group was calculated to produce blood levels of acetaldehyde slightly above those of human chronic alcoholics. There was no difference between control and experimental groups in the mean number of implantation sites in females but there was a significant dose-related increase in the number of resorptions in both treated groups over controls. At day 10 there was a slight increase in the number of developmentally retarded embryos among the treated groups. The lengths of embryos were significantly less among the treated groups, those of the 1% group being intermediate between the control and 2% group. Similarly, the protein content of the embryos was significantly less among the treated groups than among the controls. (This contrasted with the results of Henderson *et al.* (*loc. cit.*) who found a tendency towards higher protein levels in exposed groups.) By day 19 the only effect clearly related to acetaldehyde administration was a significant dose-related decrease in foetal weight. On day 10, three out of 39 'turned' embryos in the 2% group had abnormalities of the hind-limb and mid-brain regions while this was seen in only one of 59 control animals and none of the 1% group at the same stage of development. The only cardiovascular anomalies seen at day 10 were in two embryos from the 1% acetaldehyde group. Superficially, foetuses at day 19 showed no abnormalities. Acetaldehyde seems to reproduce many general effects seen when ethanol is administered to pregnant laboratory animals and this indicates that acetaldehyde may be responsible for some of the effects of ethanol.

The effects of alcohol consumption on the foetus are clearly complex and still little understood. Although it is difficult to compare the ethanol intakes in studies of such widely different design, there seems to be a correlation between the severity of the effects and the blood ethanol levels recorded. The similarity of the results obtained by Henderson *et al.* (*loc. cit.*) for acute and chronic exposure are particularly interesting. The question of the relationship between the effects and the level and pattern of exposure must be a priority for future research.

[P. Cooper—BIBRA]

ALCOHOL AND PREGNANCY—PART 2: STUDIES IN MAN

The link between severe alcohol abuse by pregnant women and the range of features that characterize the foetal alcohol syndrome (FAS) is now firmly established and the list of characteristics that are associated with the syndrome has steadily increased in recent years. However of much more general importance are the studies that are now emerging on the more subtle effects of moderate drinking.

Ninety-five cases of FAS were studied by Majewski (*Fortschr. Med.* 1978, **96**, 2207) and showed a great variety of disorders. In severe cases there was typical craniofacial dysmorphy; congenital heart disease, urogenital anomalies and anomalies of the extremities were also common. Most of the mothers were severely alcoholic. The severity of the mother's alcoholic illness seemed to be more important than the daily alcohol intake in determining the extent of the effects, suggesting that secondary factors in alcoholic illness may be teratogenic. Spiegel *et al.* (*Clin. Orthop.* 1979, **139**, 58) also found a broad range of defects in eight cases of FAS from mothers with a history of high ethanol intake. Microcephaly, prenatal and postnatal growth deficiency, developmental delay and mental retardation were all seen in seven of the eight cases.

Over a period of 3 yr, Dupuis *et al.* (*Archs Mal. Coeur* 1979, **71**, 565) studied 50 cases of heart defects in children with FAS. They considered that cardiac malformations were commonly associated with FAS, mainly taking the form of atrial and ventricular septal defects.

Habbick *et al.* (*Lancet* 1979, **I**, 580) reported three cases of FAS in children of mothers ingesting excessive amounts of alcohol. All three showed retardation of growth and of psychomotor development, microcephaly and facial features typical of FAS. Although the livers were abnormal, their histological appearance was different in each case. Thick sclerotic central veins were apparent in liver biopsies from cases 1 and 2. The hepatic abnormalities in case 3 could have been unrelated to FAS and may have resulted from cardiovascular anomalies. Factors in pregnancy other than alcohol intake may influence the severity of liver damage. Another case of liver damage in a child with FAS was seen by Møller *et al.* (*ibid* 1979, **I**, 605). This infant had an enlarged liver but normal spleen. Levels of some parameters including serum alkaline phosphatase, aspartate transferase and α-amino-nitrogen were increased. At 6 months of age the liver and biliary tract looked normal, but histological examination showed larger than normal portal spaces containing small tubular structures, with normal vessels and bile ducts. Liver disease with moderate bile-duct proliferation was diagnosed. A case of hepatoblastoma in a child with FAS was recorded by Khan *et al.* (*ibid* 1979 **I**, 1403). The transplacental alcohol may either have promoted liver cancer or have increased susceptibility to other carcinogenic substances. However this was a particularly complicated case since the mother also used illegal drugs heavily during pregnancy and the infant had a renal transplant at 7 months and was given immunosuppressive drugs to prevent rejection. Immunosuppressive therapy was another potential aetiological factor; liver cancer has developed in at least two other children after transplants.

Renal anomalies and neural-tube defects were found in a case of FAS reported by Goldstein & Arulanantham (*J. Pediat.* 1978, **93**, 636). Fuster *et al.* (*ibid* 1979, **95**, 328) also described a case of FAS in which neural-tube defects featured. Brain malformations in four infants born to two women with a heavy alcohol intake and to two others with a regular alcohol intake and occasional binge episodes were studied by Clarren *et al.* (*ibid* 1978, **92**, 64). All four brains showed similar malformations resulting from errors in the migration of neuronal and glial elements. Two of the infants had hydrocephalus. Although none of the abnormalities were unique, the patterns were unusual. On the basis of current understanding of the timing of neuronal migration, the authors considered that interference with cerebellar and brainstem development took place in the first 45 days of gestation and interference with the cerebrum took place before day 85 of gestation. Two of the four infants with ethanol-induced brain malformations did not have the external features of FAS. Thus it appears that brain damage can be the predominant effect of ethanol exposure *in utero*, and also that intermittent peak doses can cause damage.

There have been a few reports that seem to indicate that FAS may occur even when formerly alcoholic parents have given up drinking, although some authors have expressed severe doubts about the evidence. Scheiner *et al.* (*Lancet* 1979, **I**, 1077) described a case in which the father had formerly been a heavy drinker for 16 yr and the mother's drinking was intermittently heavy for a similar period. Both parents stopped drinking 18 months before conception. Their baby weighed 2215 g and was 48 cm long; she had mild facial signs of FAS and mild flat feet. Coarctation of the aorta and a small ventricular septal defect were also present. The child did well medically but was developmentally delayed both in language and in gross motor skills. She made good progress and at 32 months had normal gross motor skills and an IQ of 106. Although FAS had been indicated, FAS cases do not normally catch up in this way. The authors suggested an adverse effect of the alcohol intake on male or female germ cells before conception as one possible explanation for the child's condition. It is also possible either that the mother did drink during pregnancy or that the symptoms suggesting FAS had their origin in a different cause.

Smith & Graham (*ibid* 1979, **II**, 527), who criticized Scheiner *et al.* (*loc. cit.*) for presenting isolated, clinically-insecure cases that could lead to wrong conclusions, noted the latter's presentation of a second case of FAS at a National Foundation conference in Chicago on 27 June 1979. In this case only the father was said to have had a heavy alcohol intake at the time of conception. Smith & Graham (*loc. cit.*) did not think that either of the two cases presented had FAS; features such as postnatal catch-up in growth, normal intelligence at 32 months and coarctation of the aorta are most unusual in FAS and the faces of the children were not characteristic of the syndrome. They advocated the study of several offspring of such families and application of the term FAS only when the combined features indicated the syndrome, rather than the presentation of single, doubtful cases which might lead to incorrect conclusions that the syndrome goes beyond the alcoholic state of the mother and may involve the father.

In contrast, Véghelyi & Osztovics (*ibid* 1979, **II**, 35) felt that the effects seen by Scheiner *et al.* (*loc. cit.*) tallied with FAS, but that the most likely explanation was that the mother had been drinking during pregnancy. The only alternative explanation would be a mutagenic effect, which seemed to them to be unlikely. They had been unable to reproduce the only observation of dominant lethal mutation by alcohol in male mice (*Cited in F.C.T.* 1975, **13**, 581) and the finding had been contradicted by others. Little is known about the possible mutagenic effects on the ovum in the Graafian follicle, but these authors had found that even 1% alcohol (described as twice the human lethal dose) neither damaged cultured human lymphocytes or fibroblasts nor had a mutagenic effect. Acetaldehyde, the metabolite that may be involved in the teratogenic effects of alcohol, did not affect these cultured human cells at concentrations below 40 μmol/litre but was intensely cytotoxic and teratogenic at higher concentrations. Véghelyi & Osztovics (*loc. cit.*) provided evidence for the involvement of acetaldehyde in FAS. Eight women who had children with FAS all responded to alcohol ingestion with acetaldehyde levels three to four times the normal level, and the authors ascribe FAS to this. They also cite three cases that indicate that acetaldehyde does not affect the ovum in the follicle. Two of three cases had been alcoholics for years when they had FAS children. They then stopped drinking completely and each had a normal child. The third case had been drinking the equivalent of about 200 ml absolute alcohol daily for 10 yr when, after having two normal children, she had one with FAS. A year later she again became pregnant and 10 days later was jailed for 6 months without access to drink. She resumed drinking on her release but subsequently delivered an underweight, otherwise normal baby. When given some alcohol to drink 3 wk later, her blood acetaldehyde level rose in 30 min to about three times the normal level. [The fact that the woman had earlier borne two normal children whilst drinking heavily would seem to detract from the weight of this evidence.]

In a letter to the Editor (*Br. med. J.* 1978, **2**, 1365), Véghelyi *et al.* also stated that the blood acetaldehyde level was not dependent on the amount of alcohol consumed. The foetuses of healthy mothers would therefore be unaffected by "binge drinking" or a "couple of drinks". Indeed they considered that equivalent amounts of alcohol were consumed "with comparative impunity" by millions of women in France and Italy who took wine with their meals during pregnancy. However, if the mother's ability to metabolize acetaldehyde were defective, small amounts of alcohol would harm the child both directly and by damaging the placenta. Cutting down alcohol intake would then be useless. The alternatives are therefore either abstinence, although this would probably be broken by regular drinkers, or the measurement of prospective mothers' blood-acetaldehyde levels after a drink so that they could be advised against having a child if the level were high. Véghelyi *et al.* (*loc. cit.*) therefore discounted the conclusion in a leading article in the *British Medical Journal* (1978, **2**, 76) that since a safe

level of alcohol intake had not been established, women should cut down their drinking once they became pregnant. Work with animals supporting the view that acetaldehyde may be the agent responsible for the effects of alcohol on the foetus is reported in the previous article. It has been shown that the acetaldehyde concentration of the maternal blood after ethanol administration is higher in pregnant than in non-pregnant rats (*Cited in F.C.T.* 1975, **13,** 669). However, it has also been demonstrated that little acetaldehyde penetrates the rat foetus shortly after ethanol injection into the mother (*ibid* 1977, **15,** 77).

The 263 subjects selected by Little (*Am. J. publ. Hlth* 1977, **67**, 1154) for a study of more moderate alcohol intake were all paying members of a health maintenance organization in Seattle. They were interviewed about their smoking, use of medicines and beverage consumption, as well as their alcohol intake, during three periods: before pregnancy (the 6 months before conception) and during early and late pregnancy (the first 4 months and months 5–8, respectively). The sample was carefully selected to control for smoking, which is strongly correlated both with infant birth weight and with maternal alcohol consumption. Multiple linear regression analysis was used, regression equations being constructed for each of the three periods. The independent variables entered into the equation were maternal age, height, parity, cigarettes smoked and alcohol intake and the gestational age and sex of the child. This revealed a significant relationship between alcohol consumption before pregnancy or in late pregnancy and birth weight. The ingestion of an average of 1 oz absolute alcohol (roughly 2 oz of 100-proof whisky) daily before pregnancy was associated with a decrease in birth weight of 90·8 g and ingestion of the same amount in late pregnancy was associated with a decrease in weight of 160 g. The lack of a significant decrease in birth weight associated with alcohol consumption in early pregnancy may have been due partly to the small number of women with high alcohol intakes in this period. The authors found that alcohol consumption decreased dramatically after conception. Although birth weight is not equivalent to foetal health, reduction in birth weight associated with moderate maternal alcohol consumption may represent minimal damage to the foetus. The authors point out that the study may have been influenced by a number of factors, particularly that the sample was from a distinct population group—mainly white, middle-class women who were members of a health maintenance organization.

Hanson *et al.* (*J. Pediat.* 1978, **92**, 457) also studied the effects of moderate alcohol intake. The mothers of the 163 infants studied were again mainly white, middle-class and well educated. Women were selected who reported a daily intake of 1 oz or more of alcohol or intoxications of five or more drinks per occasion. Control infants were selected from mothers with little or no alcohol intake. Infants were evaluated for abnormalities of growth, development and morphogenesis. Eleven of the infants had features compatible with FAS and two of these were diagnosed as having the disorder (both of the mothers were alcoholics). Of the nine remaining, seven had mothers with alcohol intakes of more than 1 oz per day during the month preceding recognition of pregnancy but only one had an intake of more than 1 oz of alcohol daily during the first 5 months of pregnancy. Like Little, these authors found that, in the interests of foetal welfare, mothers decreased their alcohol intake as soon as they knew they were pregnant. In the whole group there was a significant relationship between self-reported alcohol consumption during the month preceding recognition of pregnancy and abnormalities suggesting FAS, but there was no such relationship for the first 5 months after recognition of pregnancy. No information on drinking behaviour later in pregnancy was obtained.

An experimental approach to the effects of moderate alcohol intake was taken by Lewis & Boylan (*Lancet* 1979, **I**, 388). Foetal breathing can be viewed as an index of foetal CNS arousal and is easily measured non-invasively during pregnancy. Six healthy pregnant women were given either 40 ml vodka in orange juice or the orange juice alone on two occasions between wk 34 and 38 of pregnancy. Foetal breathing was monitored using an ultrasound scanner. In the period from 30 to 60 min after the alcohol was given, the proportion of time the foetuses spent making continuous breathing movements was $14 \pm 8\%$, whereas after plain orange juice it remained at the pre-intake value of $46 \pm 16\%$. Thus ingestion of a small amount of alcohol can depress foetal CNS arousal.

According to Abel (*ibid* 1979, **II**, 105), previous workers had noted an excess of females amongst infants with FAS, but he reviewed 39 studies from Europe and the USA and found a sex ratio of 131 males to 148 females. This finding was not significant, suggesting that alcohol is no more toxic to male than to female foetuses.

There is evidence that moderate alcohol intake may affect the foetus and that brain damage may occur even when outward signs of FAS are not evident. Alcohol appears to exert its harmful effects at a very early stage of pregnancy, possibly before many women realize that they are pregnant. There is no established safe level of intake. Neither is there any real evidence that 'cutting down' is adequate—but it can do no harm. Many women already do this when they realize that they are pregnant. It would probably be of more benefit if they did so at the conception stage.

[M. A. Thompson—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD ADDITIVES AND CONTAMINANTS

Tartrazine sensitivity explored

Gerber, J. G., Payne, N. A., Oelz, O., Nies, A. S. & Oates, J. A. (1979). Tartrazine and the prostaglandin system. *J. Allergy clin. Immun.* **63,** 289.

A number of aspirin-sensitive patients are also sensitive to tartrazine (*Cited in F.C.T.* 1973, **11,** 685), although the mechanism underlying this relationship is not clear. Other nonsteroidal anti-inflammatory drugs may also provoke an asthmatic attack in aspirin-sensitive subjects, and in this case a common inhibition of prostaglandin synthesis is believed to be involved. Evidence for this comes from the *in vitro* finding that, like aspirin, such drugs inhibit the activity of cyclooxygenase, which is one of the enzymes responsible for prostaglandin formation from arachidonic acid *in vivo* (Szczeklik *et al. Br. med. J.* 1975, **1,** 67). Other enzymes involved in prostaglandin synthesis are thromboxane synthetase and acyl hydrolase, and the effect of tartrazine on all three enzymes has now been explored.

In vitro, tartrazine at concentrations of up to 30 mM had no effect on cyclooxygenase activity in microsomes from sheep seminal vesicles or guinea-pig lungs, and thromboxane synthetase activity, investigated in the latter system, was also unaffected. Control studies with indomethacin and imidazole, which are specific inhibitors for these enzymes, confirmed the validity of the test systems. Moreover, tartrazine did not prevent the aggregation of human platelets by arachidonic acid. Such aggregation is mediated by cyclooxygenase and was completely inhibited by indomethacin. The rate-limiting step in prostaglandin generation is probably the release of arachidonic acid from lipid stores by acyl hydrolase, the activity of which is stimulated by angiotensin II. Tartrazine did not inhibit the angiotensin II-stimulated release of radioactivity from rat renal papillae which had been pre-incubated with labelled arachidonic acid. Sulphanilic acid, the major urinary metabolite of tartrazine, also had no inhibitory effect on cyclooxygenase activity in sheep seminal-vesicle microsomes.

The conclusion is drawn that adverse reactions to tartrazine are unlikely to be due to inhibition of the prostaglandin pathway; patients reacting to both tartrazine and aspirin may be displaying co-existing sensitivities rather than a cross-sensitivity.

Transplacental nitrite

Globus, M. & Samuel, D. (1978). Effect of maternally administered sodium nitrite on hepatic erythropoiesis in foetal CD-1 mice. *Teratology* **18,** 367.

Nitrites have gained particular notoriety because of their ability to produce nitrosamines by reaction with secondary amines (*Cited in F.C.T.* 1974, **12,** 156). Nitrosamines have been reported to produce lymphomas in rats (Newberne, *Science, N.Y.* 1979, **204,** 1079), and may cause methaemoglobinaemia in guinea-pigs (*Cited in F.C.T.* 1975, **13,** 142). The possible embryotoxic effects of nitrites ingested by mammals during pregnancy therefore demand attention.

Pregnant mice were each given 0·5 g sodium nitrite ($NaNO_2$)/day orally by intubation for the first 14, 16 or 18 days of gestation and the effects on their offspring were studied. No significant differences in the numbers of offspring/litter, embryo weights, numbers of resorption sites/litter or foetal mortalities were observed in comparison with controls. The incidence of skeletal malformations was not significantly increased although there appeared to be a tendency towards club-feet in the treated group. However, the percentages of polychromatophilic erythroblasts and of mature erythrocytes in haemopoietic cell suspensions prepared from livers of $NaNO_2$-exposed embryos were significantly increased over controls at days 14 and 16 respectively. This was an indication of a general increase in more mature cells. According to the authors the absence of this effect at day 18 might be explained by the fact that in the mouse from days 18 to 19 the bone marrow and spleen take over from the liver as the major sites of erythropoiesis. It is conceivable that maternal exposure to $NaNO_2$ may result in foetal methaemoglobinaemia which ultimately manifests itself in an increased demand for new red cells.

Nitrosamines in bacon—mechanisms and trends

Bharucha, K. R., Cross, C. K. & Rubin, L. J. (1979). Mechanism of *N*-nitrosopyrrolidine formation in bacon. *J. agric. Fd Chem.* **27,** 63.

Havery, D. C., Fazio, T. & Howard, J. W. (1978). Trends in levels of *N*-nitrosopyrrolidine in fried bacon. *J. Ass. off. analyt. Chem.* **61,** 1379.

The presence of *N*-nitrosopyrrolidine (NPYR) in fried bacon has frequently been reported (*Cited in F.C.T.* 1975, **13,** 205; Sen *et al. Fd Cosmet. Toxicol.* 1976, **14,** 167). There has been considerable discussion of the sources of NPYR in this food (*Cited in F.C.T.* 1978, **16,** 389) and the first paper cited above further incriminates proline as the probable precursor.

The quantities of free proline detected in pork belly and raw side bacon were 11–26 and 20–81 mg/kg respectively. These values are several orders of magnitude greater than those that would be required to account for the concentrations of NPYR reported in bacon. When free proline was added to the bacon there was a proportionate increase in NPYR and this is good evidence that proline is the precursor of NPYR. The authors felt that the nitrosation of pro-

line to *N*-nitrosoproline (NPRO) followed by decarboxylation to NPYR was the most likely pathway in view of their finding that the conversion of NPRO to NPYR occurs more readily than the alternative conversion of proline to pyrrolidine. The level of NPRO in raw bacon was in the 40 mg/kg range which would account for only a small proportion of the NPYR in the fried bacon. Thus preformed NPRO in raw bacon is not the main precursor but NPRO may well be formed during cooking. The yield of NPYR is dependent upon cooking conditions. The maximum amount of nitrosamine was produced when the bacon was fried or grilled for about 12 min, and analysis of the vapours from frying bacon showed that both NDMA and NPYR increased as the cooking proceeded. This indicated that nitrosamine formation during bacon frying occurred mainly in the fat phase after most of the water was removed and therefore by a radical rather than an ionic mechanism. This point has a strong bearing on the search for suitable agents to block nitrosamine-formation in bacon. Further, the reduced yields of nitrosamines found during grilling compared with frying are explicable since the fat runs out of the heated area during grilling and never reaches the same high temperatures as during frying.

The second paper reports the results of analysis for NPYR in nine brands of bacon purchased at various intervals from 1971 to 1977 in Washington, DC. The samples were analysed both raw and after frying. No nitrosamines were found in any of the raw samples, and a downward trend was demonstrated in NPYR levels in fried bacon. In 1971 three different brands contained mean levels of 73–104 ppb ($b = 10^9$) NPYR, and in 1972 the mean levels in five brands ranged between 13 and 108 ppb. However in 1977 the range was 5–75 ppb NPYR. In six brands NPYR concentration significantly declined over the period 1971–2 to 1972–4 and in five of these six brands and in one other there was little change in the NPYR concentration over the period 1974–7.

Although sampling was limited to one city, it appears that the decreased levels of NPYR are partially explained by the use of reduced nitrite levels and increased ascorbate levels in bacon-curing mixes. However, any further reduction of NPYR may prove difficult to achieve if the growth of *Clostridium botulinum* is still to be controlled. Compounds other than nitrites might conceivably be incorporated into bacon to achieve this aim.

Raw soya flour and nitrosamines—a potent mixture for rats

Levison, D. A., Morgan, R. G. H., Brimacombe, J. S., Hopwood, D., Coghill, G. & Wormsley, K. G. (1979). Carcinogenic effects of di(2-hydroxypropyl)nitrosamine (DHPN) in male Wistar rats: promotion of pancreatic cancer by a raw soya flour diet *Scand. J. Gastroent.* **14**, 217.

Raw soya beans contain a number of heat-labile substances that inhibit the activity of proteolytic digestive enzymes and stimulate pancreatic protein synthesis and enzyme secretion. These effects generally lead to enlargement of the pancreas and growth inhibition, although the response to feeding raw soya beans varies in different species (Rackis *et al. J. Am. Oil Chem. Soc.* 1979, **56**, 162). The authors cited above previously reported that feeding raw soya flour to rats produced hypertrophic and adenomatous nodules of the acinar cells of the pancreas and had a potentiating effect on the action of the pancreatic carcinogen azaserine. They have now reported on the combined effects in rats of feeding raw or heated soya-bean flour and administering di-(2-hydroxypropyl)nitrosamine (DHPN) by injection. DHPN has been shown to be a potent pancreatic carcinogen in hamsters (Pour *et al. J. natn. Cancer Inst.* 1975, **54**, 141) but has only weak carcinogenic activity in the rat pancreas (Mohr *et al. ibid* 1977, **58**, 361).

Thirty-two male Wistar rats, 3–4 months old, were fed raw soya flour (RSF) and a further 32 were given soya flour that had been heat treated (HSF) to inactivate the trypsin inhibitor present in the raw flour. Half the rats in each of these dietary groups were given weekly ip injections of DHPN (0·2 mg/rat for 4 wk and then 0·1 mg/rat for a further 20 wk) while the remainder were given saline ip. All the rats receiving DHPN died or had to be killed within 16 months of the start of the experiment, and those given saline were killed in similar numbers at regular intervals over 2 yr.

Amongst the rats injected only with saline, the pancreases of those fed RSF weighed more and were larger than those of rats fed HSF and were found to contain numerous nodules 1–10 mm in diameter. These nodules consisted of enlarged acinar cells, with large pleomorphic nuclei and many mitotic figures, some of which were abnormal. The majority of the acinar cells contained increased amounts of zymogen although those in some nodules contained very little. Most of the nodules simply compressed the surrounding pancreatic tissue (hyperplastic nodules), but some were adenamatous, having a well-defined fibrous capsule. Only four animals in the group given HSF had pancreatic acinar nodules. These were all hyperplastic, and were smaller and less numerous than those in the RSF-fed rats.

In the groups injected with DHPN, the pancreases of six of the 16 rats fed HSF contained occasional microscopic hyperplastic nodules. In contrast, the 13 pancreases that were fit for examination in the RSF-fed group had a profusion of such nodules; there were macroscopic hyperplastic nodules in ten of these organs and adenomatous nodules in two. In addition the pancreas of one RSF-fed rat contained an adenoma, apparently arising from the ductal epithelium, and pancreatic adenocarcinomas were present in two animals in this group. Carcinomas at other sites were common in all the rats injected with DPHN.

The results indicate that by inducing pancreatic growth, RSF potentiates the otherwise weak carcinogenic effect of DHPN in the rat pancreas. The combination of feeding RSF and injecting DHPN resulted not only in a greater incidence and earlier appearance of pancreatic nodules than in the rats given a combination of DHPN and HSF or RSF alone, but also in the induction of two pancreatic adenocarcinomas. However DHPN appears to inhibit the growth-promoting effects of RSF to some degree, since pancreatic hypertrophy was significantly less in the rats given RSF and DHPN than in those given RSF and

saline. Such inhibition of the hypertrophic effects of RSF was not observed with azaserine in an earlier study by this group.

It remains to be established whether the pancreatic acinar nodules produced by feeding RSF would, without additional treatment, undergo malignant change. The authors suggest, however, that the RSF-fed rat appears to be a suitable model for screening for environmental pancreatic carcinogens, since the effects of both azaserine and DHPN have been potentiated by the ingestion of this type of diet.

AGRICULTURAL CHEMICALS

The non-teratogenicity of dibromochloropropane

Ruddick, J. A. & Newsome, W. H. (1979). A teratogenicity and tissue distribution study on dibromochloropropane in the rat. *Bull. envir. Contam. Toxicol.* **21,** 483.

1,2-Dibromo-3-chloropropane (DBCP) has not only shown carcinogenic and mutagenic activity in experimental studies, but has caused infertility in male workers exposed to it (*Cited in F.C.T.* 1976, **14,** 505; *ibid* 1979, **17,** 555. Its effects on the developing foetus are the main subject of the present study.

Groups of 15 pregnant rats were given 12·5, 25·0 or 50·0 mg DBCP/kg in corn oil by gavage on days 6–15 of gestation, and autopsies were conducted on day 22. The two higher doses significantly depressed maternal weight gain, and at the highest level foetal weight was also significantly reduced in comparison with that in the corn-oil treated controls. No foetuses were visible in four of the females given 50 mg/kg, but in three of these the uteri were oedematous and contained a pinkish fluid. However, no increase in skeletal or visceral anomalies was observed at any level of treatment.

In pregnant females treated in the same way at a dose level of 25 mg/kg and killed 1, 3, 6, 12 or 24 hr after the last dose, DBCP was found in all tissues examined (foetus, spleen, brain, heart, lung, kidney, liver, fat and blood). The highest levels were reached after 3 or 6 hr, the fat containing by far the greatest concentration (5·38 ppm). After 12 hr, this was the only tissue in which significant levels still persisted, and by 24 hr these levels had declined to 0·170 ppm.

Methoxychlor carcinogenicity

Reuber, M. D. (1979). Interstitial cell carcinomas of the testis in Balb/c male mice ingesting methoxychlor. *J. Cancer Res. clin. Oncol.* **93,** 173.

Reuber, M. D. (1979). Carcinomas of the liver in Osborne-Mendel rats ingesting methoxychlor. *Life Sci.* **24,** 1367.

The organochlorine pesticides have been widely used in agriculture, although in many countries their use has been restricted because of concern about their persistence in the environment. DDT, a well-known member of this group of pesticides, induced hepatic changes in rodents and these changes may progresss to tumour formation in some species, particularly the mouse, although there is debate about whether the tumours are malignant (*DDT and its Derivatives. Environmental Health Criteria* 9, p. 17; WHO, Geneva, 1979). Methoxychlor is an organochlorine closely related to DDT. No evidence of carcinogenicity emerged from an NCI bioassay in which Osborne-Mendel rats or B6C3F1 mice were fed up to 1385 or 3491 ppm methoxychlor, respectively, in the diet (*Federal Register* 1978, **43,** 11760). Neither did methoxychlor prove to be carcinogenic in rats in three earlier studies, in one of which levels of up to 1600 ppm were used (*IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man.* Vol. 5. *Some Organochlorine Pesticides*, p. 193; IARC, Lyon, 1974). In contrast, in the above-cited studies carried out at FDA laboratories, methoxychlor was found to be carcinogenic in both Balb/c mice and Osborne-Mendel rats.

In a 2-yr feeding study, 100 male and 100 female mice of the Balb/c and C3H strains were given diet containing 750 ppm technical methoxychlor or 100 ppm technical DDT. Interstitial-cell carcinomas of the testis developed in 53% of the male Balb/c mice ingesting methoxychlor, but in only 11% of the controls. The tumours that developed in the treated mice were more malignant, appeared at an earlier age and were larger, less histologically differentiated and more invasive than in the controls.

Testicular carcinomas did not develop in Balb/c mice given DDT or in C3H mice given either pesticide. The author suggests that the carcinogenic effects of methoxychlor on the testes of Balb/c mice may be related to the oestrogenic activity of the compound (Nelson *et al. J. Toxicol. envir. Hlth* 1978, **4,** 325).

It is also reported that C3H strain male and female mice given methoxychlor or DDT developed more liver tumours than the Balb/c mice and that the Balb/c male mice also had lung tumours. However details of these observations are not given in the paper discussed here.

In another 2-yr study, male and female Osborne-Mendel rats were fed 0, 10, 25, 100, 200, 500 or 2000 ppm methoxychlor in the diet. Ten out of 52 treated female rats and eight out of 32 treated male rats had hyperplastic nodules or carcinomas of the liver, compared with the rate of one in 50 generally observed in this strain of rat at the laboratory concerned. Hyperplastic nodules occurred at all dose levels in the females, but only at 100, 500 and 2000 ppm in the males. Liver carcinomas developed only in the rats given 2000 ppm methoxychlor—occurring in three out of nine females and three out of eight males. In female rats the incidence of ovarian neoplasms and carcinomas was also significantly increased.

[The changes induced by organochlorines in the livers of rodents appear to be peculiar to those

species, and are similar to those caused by phenobarbital. In the WHO publication already mentioned (*DDT and its Derivatives*, p. 114; WHO, Geneva, 1979), it is concluded that all available evidence indicates that man does not appear to be susceptible to the tumorigenic action of the organochlorine insecticides and phenobarbital. It is suggested that attention should be turned from the narrow question of tumorigenicity of DDT and phenobarbital in the liver of mice and rats to the broader basis of their action.]

OCCUPATIONAL HEALTH

Signs of cadmium toxicity

Prigge, E. (1978). Early signs of oral and inhalative cadmium uptake in rats. *Arch. Tox.* **40,** 231.

There have been numerous studies of the toxicity of cadmium (Cd) and of its effects on the kidney (*Cited in F.C.T.* 1979, **17,** 84), the testis (*ibid* 1979, **17,** 544), the prostate (*ibid* 1979, **17,** 546) and the lung (*ibid* 1978, **16,** 288). Although anaemia has been reported to be one of the first signs of toxicity following Cd ingestion (Freeland & Cousins, *Nutr. Rep. Int.* 1973, **8,** 337), it has been found that exposure to Cd aerosols did not significantly lower serum Fe levels in adult rats (Prigge, *Toxicology* 1978, **10,** 297). The study cited above was aimed to further investigate serum Fe levels and other early signs of toxicity in rats given Cd orally or by inhalation.

In the inhalation experiment female rats were exposed to cadmium oxide aerosols at levels of 25 or 50 μg Cd/m^3 for 90 days or of 100 μg Cd/m^3 for 63 days. Other rats were given 25, 50 or 100 ppm Cd (as chloride) in their drinking water for 90 days. The two larger doses, administered by either route, reduced body weight compared with the controls. Five of the 12 rats inhaling 100 μg Cd/m^3 died between days 45 and 60. Inhalation was associated with a dose-related increase in lung weight, but no such change accompanied ingestion. Inhalation at the 100 μg Cd/m^3 level resulted in decreased blood pH, decreased pO_2, and increased pCO_2. Although inhalation did not alter serum Fe concentration, ingestion significantly decreased it. There was a dose-dependent increase in haemaglobin levels and haematocrit values after inhalation but not after ingestion of Cd. Inhalation of Cd did not significantly alter urinary protein output, but proteinuria increased in the rats given 50 or 100 ppm Cd by ingestion. Neither route altered alkaline phosphatase activity in the urine, but oral administration lowered it in serum. Liver concentrations of Cd were much higher after oral exposure than after inhalation, but kidney concentrations were similar.

The lungs of rats exposed to Cd aerosol showed emphysema, and cell proliferation was evident in bronchi, bronchioles and alveoli. Xanthoma cell areas and histiocytic cell granulomas were seen, but there was no oedema. The animals' livers were normal, and their kidneys showed only the occasional tubular swelling.

In this study, after Cd inhalation pulmonary changes evidently preceded renal damage. The authors contrasted their findings with those of an epidemiological survey. Lauwerys *et al.* (*Archs envir. Hlth* 1974, **28,** 145) found that kidney damage was more prevalent than pulmonary ventilatory changes, although they pointed out that the methods used were more sensitive in revealing kidney lesions than an impairment of lung function. The differences between the results of these two studies may be due to species differences or to the much longer exposure periods and differences in doses in Lauwerys' investigations. On the other hand, a significant fraction of the Cd inhaled by humans may be absorbed orally, thus facilitating earlier renal damage.

Nickel and tracheal transplants

Yarita, T. & Nettesheim, P. (1978). Carcinogenicity of nickel subsulphide for respiratory tract mucosa. *Cancer Res.* **38,** 3140.

Nasal and lung cancers (and possibly laryngeal cancers too) are associated with the industrial handling of nickel compounds (*Cited in F.C.T.* 1972, **10,** 113; *ibid* 1974, **12,** 428). Nickel subsulphide (Ni_3S_2) has been shown to induce neoplasms in rats after intrarenal or intramuscular injection (*ibid* 1977, **15,** 370). Experiments with hamster tracheae have indicated that nickel exposure lowers ciliary activity and leads to epithelial damage (*ibid* 1980, **18,** 103). The paper cited above describes the effect of pellets of Ni_3S_2 on transplanted rat tracheae.

Tracheae tied to polyethylene tubing were transplanted beneath the dorsal skin of female rats (two tracheae per recipient). The transplants were implanted with gelatin pellets containing 1 or 3 mg Ni_3S_2, and two or three tracheae were removed from their hosts for histological examination at intervals of 1 wk to 12 months. After one month about 50% of the sulphide had disappeared from the tracheae, and after 3 months 85% had gone. After 5 months about 0·1 mg of Ni_3S_2 remained in the tracheae of both groups. Control pellets of gelatin produced only transient mild epithelial hyperplasia during the first 2 wk. With 1 mg implants of Ni_3S_2, minor epithelial changes comprising generalized hypertrophy with tall columnar cells and small isolated areas of hyperplasia, and diffuse lymphocytic infiltration of sub-mucosa, appeared early. After one and 2 months atrophy and epithelial flattening predominated, and after 3 months there was little difference from control epithelium apart from a few small patches of atrophic and thin squamous epithelium. In tracheae implanted with 3 mg Ni_3S_2 the early appearance of multilayered undifferentiated epithelium with patches of atrophic epithelium and focal squamous metaplasia gave way at 2–4 wk to very large irregular cells with giant nuclei and focal epithelial necrosis; particles assumed to be the sulphide were seen in connective tissue. The submucosa showed a general increase in cellular ele-

ments, and heavy lymphocytic infiltration. The submucosa contained more fibroblasts, capillaries, and what appeared to be myoblasts. After 2–4 months atrophy predominated, but there were some hyperplastic and metaplastic foci. At 5 months changes in the epithelial morphology were slight, but the submucosal tissue increased dramatically in bulk and constricted and later occluded the lumen after 7 to 9 months.

In a separate study of tumour induction, transplanted tracheae implanted with pellets containing 1 or 3 mg Ni_3S_2 or control pellets were examined at intervals for 20 months. With 1 mg Ni_3S_2, tumours first appeared at 11 months; in all 60 tracheae there were three sarcomas and six carcinomas. With 3 mg Ni_3S_2, tumours first appeared at 9 months; in 64 tracheae there were 44 sarcomas and one squamous carcinoma. Although the toxicity of Ni_3S_2 may mask its carcinogenicity, the authors suggest that tracheal epithelium may be relatively resistant to nickel carcinogenicity and that additional factors may be involved in the aetiology of respiratory tract cancer in nickel workers.

[The significance of these results is extremely difficult to evaluate in the light of the elaborate experimental design that was used.]

Predicting pathogenicity of mineral dusts

Chamberlain, M., Brown, R. C., Davies, R. & Griffiths, D. M. (1979). *In vitro* prediction of the pathogenicity of mineral dusts. *Br. J. exp. Path.* **60,** 320.

Exposure to airborne mineral dusts is associated with the development of various lung diseases in man, and the effects of fibrous and non-fibrous dusts and of fibre morphology have been much investigated (*Cited in F.C.T.* 1971, **9,** 594). These effects and the mechanisms of action of such dusts have been studied extensively in laboratory animals (*ibid* 1973, **11,** 338; *ibid* 1974, **12,** 591) and in cell cultures (Allison, in *Inhaled Particles. III*, edited by W. H. Walton; p. 437; Unwin Brothers Ltd.,Woking, 1971). In the study cited above the activities of eight mineral dusts against macrophages and against two non-macrophage cell lines were investigated and compared with their abilities to induce fibrosis and mesothelioma in rats by intrapleural implantation.

The dusts used were six samples of fibrous Dawsonite (dihydroxy aluminium carbonate) of different fibre-size distributions, non-fibrous Dawsonite, and Fybex (potassium octatitanate). They were tested in a mouse peritoneal-macrophage culture for their ability to induce macrophage enzyme release, while their activity in non-macrophage cell cultures was assessed from their ability to induce giant-cell formation in a human tumour-cell line (A549) of Type II alveolar cell origin and from their effect on the survival of Chinese hamster V79-4 cells. The results were compared with the *in vivo* findings of other workers, who investigated the effects of intrapleural implantation of the same eight dusts on the induction of mesothelioma and fibrosis in rats. The estimates of *in vivo* tumorigenicity were based on single-dose experiments, however, and therefore could only be used as a crude measure of the activities of the dusts.

In all three *in vitro* tests, the dusts showing the least activity were the non-fibrous Dawsonite and a sample of fibrous Dawsonite in which all the fibres were less than 5 μm long. These two dusts also showed the least activity in the studies *in vivo*.

Correlation coefficients were calculated for the number of fibres in certain size ranges against dust activity in both the *in vivo* and *in vitro* systems. The results gave some indication that all these biological systems responded to fibres of similar sizes, the long fine fibres ($\geqslant 10$ μm long and $\leqslant 1{\cdot}4$ μm diameter), and there was evidence of a quantitative response to long fibres rather than of a graduation of activity with length. It was concluded that the *in vitro* systems used in the study could form the basis of a screening system for the detection of fibrogenic and carcinogenic mineral dusts.

Ethylene oxide: more cause for concern

Gross, J. A., Haas, M. L. & Swift, T. R. (1979). Ethylene oxide neurotoxicity: report of four cases and review of the literature. *Neurology, Minneap.* **29,** 978.

Ethylene oxide (EO) is used as a precursor for many industrial chemicals and is also used for sterilizing hospital equipment. However, the safety of EO has been questioned following reports that it has mutagenic effects in rats and also in humans who have been accidentally exposed to high concentrations of the gas (*Federal Register* 1978, **43,** 3800). EO has been linked with an increased incidence of leukaemia among workers sterilizing medical supplies in a Swedish factory (*Cited in F.C.T.* 1979, **17,** 686), and there have also been reports that EO produces haematological effects, hypersensitivity and neurotoxicity (*Federal Register* 1978, **43,** 3800). Further evidence of neurotoxicity now comes from case studies of four workers exposed to EO.

The four men, aged between 27 and 31 yr, operated a medical-supplies sterilizer that was found to have leaked during the 2 months following its installation. Over this period, or shortly afterwards, the operators had neurological disorders. One had acute central nervous system (CNS) symptoms, but no evidence of peripheral nervous system (PNS) involvement. He worked with the leaking sterilizer for up to 70 hr/wk for 3 wk and since he was new to the job he probably spent longer than the others carrying out loading and unloading tasks involving peak EO exposure. Two of the other workers, exposed for 2 or 3 wk, had milder CNS symptoms but also had symptoms of peripheral neuropathy. The fourth worker had been exposed for 2 months, but had no symptoms of either CNS or PNS abnormalities. However, nerve-conduction studies showed sensorimotor polyneuropathy. This worker was said to be the most efficient and to be the fastest at loading and unloading the sterilizer. EO-exposure levels were not monitored, but all the workers intermittently smelled the gas, which probably indicated a level of more than 700 ppm.

In all symptomatic cases there was a marked subjective improvement within 2 wk of the end of EO exposure. When examined after 2 months, no CNS, PNS or nerve-conduction abnormalities were found in the man who had shown acute CNS symptoms,

even though he returned to work as a sterilizer operator, under conditions of lower EO exposure. Two of the three workers who had peripheral neuropathy also returned to work under conditions of lower EO exposure, but nerve-conduction studies showed no improvement while the third returned to a job in which he was not exposed to EO and nerve-conduction studies showed a significant improvement.

A safe level for inhaled furfural?

Feron, V. J., Kruysse, A. & Dreef-van der Meulen, H. (1979). Repeated exposure to furfural vapor: 13-week study in Syrian golden hamsters. *Zentbl. Bakt. Hyg. B* **168,** 442.

Hamsters exposed to 250–400 ppm furfural daily for 52 wk showed growth retardation and a number of local toxic effects, including irritation of the nasal mucosa and atrophy of the olfactory epithelium (Feron & Kruysse, *Toxicology* 1978, **11,** 127). The effect of lower concentrations has now been investigated.

Syrian golden hamsters were exposed to mean concentrations of 20, 115 or 552 ppm furfural (77, 448 or 2165 mg/m^3 air) for 6 hr/day on 5 days/wk for 13 wk. At the highest level there was evidence of eye and nose irritation and slight growth retardation, accompanied in males by an increase in serum glutamic–pyruvic transaminase activity and a possible increase in relative liver weight. Pathological changes were confined to the nasal cavity, and in rats exposed to 552 ppm they consisted of focal atrophy and hyperplasia of the olfactory epithelium, with accumulations of sensory cells and cyst-like structures formed in the lamina propria from infolded epithelium. Slight atrophy and hyperplasia of the olfactory epithelium were the only effects observed at 115 ppm, and at 20 ppm no changes could be detected.

It was concluded in the light of this study that the current TLV of 5 ppm (20 mg/m^3) recommended for furfural by ACGIH is adequate. However, the fact that some pathological changes were found in the nasal mucosa of hamsters exposed to 115 ppm suggests that furfural may contribute to the irritancy of tobacco smoke, in which levels of 40–100 ppm have been reported.

[A review of furfural toxicity (Opdyke, *Fd Cosmet. Toxicol.* 1978, **16,** 759) notes that Russian papers have reported decreases in blood cholinesterase and serum albumin, increased globulin levels and changes in motor chronaxy in rats exposed to furfural concentrations of only 10 mg/m^3 or less. As these parameters were not investigated in the current study, the validity of the TLV could still be disputed.]

Bladder tumours from nitrosodiphenylamine

Cardy, R. H., Lijinsky, W. & Hildebrandt, P. K. (1979). Neoplastic and nonneoplastic urinary bladder lesions induced in Fischer 344 rats and B6C3F1 hybrid mice by *N*-nitrosodiphenylamine. *Ecotoxic. envir. Safety* **3,** 29.

N-Nitrosodiphenylamine (NDPA), used in rubber vulcanization, gave no evidence of carcinogenicity in previously reported long-term oral and injection studies in rats (*Cited in F.C.T.* 1968, **6,** 648 & 670), the former study involving the administration of 120 mg/g for 700 days. It was also non-mutagenic in *Neurospora crassa* (Marquardt *et al. Naturwissenschaften* 1963, **50,** 135) and *Salmonella typhimurium* (Yahagi *et al. Mutation Res.* 1977, **48,** 121). Now, however, comes a report that NDPA has produced bladder tumours and other lesions in rats and mice.

NDPA (97·8% pure) was administered for 2 yr initially at dietary levels of 2000 and 4000 ppm to rats, 10,000 and 20,000 ppm to male mice and 5000 and 10,000 ppm to female mice, although because of toxicity and levels given to the female mice had to be lowered to 1000 and 4000 ppm after 38 wk. Weight loss in these female mice was severe, whereas in other groups weight throughout was only 10–20% below that of the controls. Survival was reduced significantly only in female rats.

After 2 yr there was a very high incidence of transitional-cell carcinoma of the bladder in rats in the high-dose group, 38% of the males and 86% of the females being affected while no such tumours occurred in the controls. Rats on the lower dose developed no carcinomas but in some cases showed epithelial hyperplasia of the bladder, which was the first stage of the neoplastic development seen at the high dose level. Transitional-cell carcinomas of the bladder occurred in only 2% of the mice (both male and female) on the low dose and in none of those on the higher dose, but chronic submucosal inflammation was common in all four treated groups, its incidence at the upper dose level reaching 67% in males and 79% in females. There was also a dose-related increase in epithelial hyperplasia, which occurred in 15% of high-dose males and 13% of high-dose females. Transitional-cell papillomas were found in one mouse from each group of approximately 50 treated males.

The maximum daily intake of NDPA by the rats was calculated to be 320 mg/kg for females and 240 mg/kg for males, whereas only 120 mg/kg was given in the previous negative study. This difference in dose levels could account for the different results. However, NDPA has been regarded as non-carcinogenic because it lacks oxidizable hydrogens on the carbon atoms adjacent to the *N*-nitroso group, rendering it insusceptible to activation to an alkylating moiety. It is suggested, therefore, that in this study NDPA may have acted as a trans-nitrosating agent, reacting with an amine in the food to form another nitrosamine which then gave rise to bladder carcinomas. Unfortunately the diet used was not analysed for nitrosatable amines, and hence no guess could be made as to the responsible agent.

[The paper does not identify the impurities that accounted for 2·2% of the test material, and it is perhaps a possibility that some contaminant was involved in the induction of these bladder tumours.]

Solvent cocktails

Angerer, J. & Lehnert, G. (1979). Occupational chronic exposure to organic solvents. VIII. Phenolic compounds—metabolites of alkylbenzenes in man.

Simultaneous exposure to ethylbenzene and xylenes. *Int. Archs occup. envir. Hlth* **43,** 145.

Savolainen, H., Vainio, H., Helojoki, M. & Elovaara, E. (1978). Biochemical and toxicological effects of short-term, intermittent xylene inhalation exposure and combined ethanol intake. *Arch. Tox.* **41,** 195.

In industry, exposure of workers to solvents usually involves more than one compound, and investigations have been carried out to assess the effects of combined exposure. It has been shown that simultaneous exposure of rats to 1,1,1-trichloroethane and trichloroethylene enhances microsomal drug-metabolizing enzyme activity (*Cited in F.C.T.* 1980, **18,** 206). Other factors may also affect the metabolism of solvents. For instance, ethanol potentiates the effect of trichloroethylene on the central nervous system, liver and kidney, and in human volunteers who inhaled trichloroethylene it was found that administration of ethanol increased the level of the unmetabolized solvent in the blood (*ibid* 1976, **14,** 511). Inhalation of xylene alone at levels of 78 and 780 ppm had little effect on rats, guinea-pigs, squirrel monkeys and beagle dogs (*ibid* 1971, **9,** 598). However, in combination with other inhaled solvents or with ethanol ingestion, xylene may not be so innocuous.

The effects of occupational chronic exposure to xylenes and ethylbenzene are described in the first paper cited above. The blood levels of the solvents and the 24-hr urinary excretion of phenolic metabolites were determined in four female laboratory assistants who were exposed to a solvent mixture containing 52·63% *m*-xylene, 25·25% ethylbenzene, 15·24% *p*-xylene, 6·72% *o*-xylene and 0·16% toluene. The mean atmospheric concentrations of the solvents in the working area of three of the assistants (A, B and C) were 70 ppm (*m* + *p*)-xylene, 42 ppm ethylbenzene and 12 ppm *o*-xylene, while in the working area of the fourth assistant (D) the atmospheric solvent concentrations were slightly lower, being 61, 37 and 11 ppm, respectively. At the end of the working day, which followed 2 days when the women were not exposed to the solvent mix, the mean solvent concentrations in the blood of assistants A, B and C were 1·40 mg (*m* + *p*)-xylene/litre, 0·76 mg ethylbenzene/litre and 0·20 mg *o*-xylene/litre. The levels of these solvents in the blood of assistant D were 1·04, 0·50 and 0·11 mg/litre, respectively. About 1·1–1·4% of the retained ethylbenzene was metabolized to 2-ethylphenol and excreted in the urine during the 4 hr following the beginning of the exposure. However, 2,4-dimethylphenol, which has been reported to be a metabolite of *m*-xylene, was not detected. It seemed therefore that, under conditions of simultaneous exposure, competition between ethylbenzene and the xylenes for enzymatic oxidation of the aromatic nucleus prevented the oxidation of the xylenes. The question of carcinogenicity is raised by the possibility that aromatic epoxides are formed as intermediates in the metabolism of ethylbenzene to 2-ethylphenol, but this aspect is not considered in any detail in this paper.

The second study cited above deals with the combined effects of ethanol ingestion and xylene inhalation in rats. Twenty adult male Wistar rats were exposed to an atmospheric xylene level of 12·3 μmol/litre (1·3 mg/litre or 300 ppm) for 2 wk (6 hr/day; 5 days/wk). Ten of these xylene-exposed rats and ten out of 20 control rats were given 15% (v/v) aqueous ethanol as their only liquid source; the rest of the animals were given water. Inhalation of xylene caused a marked accumulation of the solvent in the perirenal fat, but after 2 wk the xylene level in this fat was significantly less in the group given xylene and ethanol than it was in the rats given xylene alone. Also at wk 2 a potentiation of renal and, to a lesser extent, hepatic microsomal ethoxycoumarin *O*-deethylase activity was demonstrated in the rats that were both ingesting alcohol and inhaling xylene. Lysosomal acid-proteinase activity in the brains of rats inhaling xylene was significantly greater than that in the controls after 1 wk, but after 2 wk significantly increased proteinase activity was only evident in the group exposed to both xylene and ethanol. Ethanol also potentiated the behavioural effects of xylene inhalation; in open-field behavioural studies carried out 1 hr after the end of exposure on day 4, increased rearing frequencies were observed in the rats exposed to both xylene and ethanol.

ENVIRONMENTAL CONTAMINANTS

More on the foetal effects of chromium

Gale, T. F. & Bunch, J. D., III (1979). The effect of the time of administration of chromium trioxide on the embryotoxic response in hamsters. *Teratology* **19,** 81.

Last year we published an abstract of a preliminary investigation of the teratogenic effects of chromium trioxide in the hamster (*Cited in F.C.T.* 1979, **17,** 417). The authors cited above have now reported the results of further investigations on this compound, designed this time to determine the effects of the time of exposure of the females on embryotoxicity.

Groups of six Charles River Lakeview golden hamsters were given a single iv dose of 8 mg chromium trioxide/kg on day 7, 8, 9, 10 or 11 of gestation. Control animals were treated with demineralized-distilled water. The hamsters were killed on day 15 of gestation, the numbers of resorption sites were noted, and the foetuses were examined for abnormalities.

Maternal toxicity was evident in many of the animals, and was most severe in the females treated with chromium on day 7 of gestation, becoming progressively less noticeable in those dosed later in pregnancy. The maternal toxicity was characterized by weight loss, by mottled kidneys showing varying degrees of tubular necrosis and in some cases by gall bladders distended with bile. The frequency of resorptions was greater in females treated on day 7 of pregnancy and decreased progressively in those treated on day 8, 9, 10 or 11. Abnormalities were observed only in the foetuses of females treated on day 7, 8 or 9. The predominant malformation was cleft palate, the incidence

of which was greatest in foetuses of dams treated on day 9.

Normal palatogenesis in the hamster begins on day 12 of pregnancy and is completed early on day 13. Therefore, assuming that chromium acts directly on the differentiating tissues, the lack of effect of chromium administered on days 10 and 11 is surprising. The authors suggest that at the dose level used in the experiment, it may have taken several days for the amount of chromium required to disrupt palatogenesis at the appropriate time to accumulate in the palatal region. A study of the severity of the abnormality indicated that while there was considerable variation in the degree of palatal development, the majority of the abnormal palates had reached a relatively late stage of development—that at which the middle and posterior thirds of the hard palate were used.

There were indications in this study that a delay in foetal growth may be a major factor in chromium-induced cleft palate. The crown-rump lengths of chromium-exposed foetuses with cleft palates were significantly shorter than those of chromium-exposed foetueses without palatal abnormalities. Furthermore, the foetuses with normal palates from treated females had shorter crown–rump lengths than the corresponding controls, although the difference in size was only significant in foetuses from dams treated on days 7 and 10. However the site of action of chromium in producing embryotoxicity is still in need of clarification.

Foetal effects of methylmercury and cadmium

Grady, R. R., Kitay, J. I., Spyker, J. M. & Avery, D. L. (1978). Postnatal endocrine dysfunction induced by prenatal methylmercury or cadmium exposure in mice. *J. envir. Path. Toxicol.* **1**, 187.

Fuyuta, M., Fujimoto, T. & Hirata, S. (1978). Embryotoxic effects of methylmercuric chloride administered to mice and rats during organogenesis. *Teratology* **18**, 353.

Both in man and in animals Hg is transferred across the placenta and accumulates in foetal tissues (*Cited in F.C.T.* 1978, **16**, 622). In animals, particularly high levels of Hg are found in the foetal brain. In addition, perinatal carbohydrate metabolism has been shown to be deranged in the offspring of rats exposed briefly to low doses of methylmercury (MeHg) during gestation (Snell *et al. Toxicology* 1977, **8**, 277). In the first study cited above, the effects of MeHg exposure on the pituitary–adrenal axis were examined.

Mice were given orally 2 mg MeHg dicyandiamide/kg or 2 mg $CdCl_2$/kg on days 15, 17 and 19 of gestation and their offspring were exposed to MeHg and Cd *via* milk from mothers that were dosed on alternate days after delivery until day 20. When they were between 460 and 480 days old the offspring exposed to MeHg and the corresponding controls were killed and examined. Some of the Cd-exposed mice were killed on day 277, and the rest were killed between days 460 and 480. Male offspring showed a 30% reduction and females a 20% reduction in liver weight after exposure to MeHg. In males, total liver capacity to metabolize corticosterone was impaired, but in females only the metabolism of the corticosterone side chain was significantly reduced. Adrenal weight was unchanged in both sexes. Corticosteroid production was not significantly impaired but there were indications that MeHg did affect adrenal secretory capacity although the wide variation in results did not permit a firm conclusion to be drawn, and no alteration in plasma corticosteroid concentration was detected.

Cd-exposed female offspring showed significant increases in body and liver weights after 277 days, but no effects were observed in males at this age. At 460–480 days of age a sex difference in response to Cd was noted. In females there was a doubling of adrenal steroidogenesis and a significant increase in plasma corticosterone concentration, while in males the major effect was enhancement of hepatic reductive capacity.

The second paper describes the effects of giving methylmercuric chloride orally to mice on days 6–13 of gestation at dose levels of 7·5, 6·0, 5·0 or 2·5 mg/kg/day. Foetuses were examined on day 18. In a parallel experiment pregnant rats were given 7·5, 5·0 or 2·5 mg MeHgCl/kg/day from day 7 to day 14 of gestation and foetuses were examined on day 20. Treatment with 7·5 mg MeHgCl/kg caused death or resorption in 98·7% of foetuses in mice and 42·4% of foetuses in rats. At 6·0 mg MeHgCl/kg foetal death occurred at a rate of 34·2% in mice, with a significant decrease in mean weight of male foetuses and a markedly increased incidence of malformations. In rats at 5·0 mg MeHgCl/kg foetal weight decreased and the malformation rate increased. The commonest malformations seen were cleft palate and fused thoracic vertebrae in mice, and cleft palate, generalized oedema, brain lesions and wavy ribs in rats.

Hormonal effects of phthalic acid esters

Oishi, S. & Hiraga, K. (1979). Effect of phthalic acid esters on gonadal function in male rats. *Bull. envir. Contam. Toxicol.* **21**, 65.

Phthalic acid esters are used as plasticizers for a wide range of synthetic polymers. Di-(2-ethylhexyl)-phthalate (DEHP) is the phthalate most extensively used in polyvinyl chloride manufacture, and is known to be widely distributed in the environment. DEHP has a low acute toxicity in experimental animals but it has been reported to have antifertility and mutagenic effects in mice at high dose levels (*Cited in F.C.T.* 1975, **13**, 587) and its administration to rats had been shown to cause testicular atrophy (Gray *et al. Fd Cosmet. Toxicol.* 1977, **15**, 389). In the study cited above hormonal changes occurring in the rat testis after DEHP treatment were investigated.

Male Wistar rats were injected ip with 1·25 g DEHP/kg/day for 5 days. Control rats were injected with olive oil. Testicular venous blood was collected from about half the rats in both the treated and control groups 24 hr after the last injection. The remaining animals were given 100 IU human chorionic gonadotrophin (HCG) by iv injection and testicular venous blood was collected 15 min later. The blood samples were centrifuged and the testosterone concen-

tration in the plasma was determined fluorometrically.

Plasma testosterone concentrations were significantly lower in DEHP-treated rats than in the controls both before and after the administration of HCG, although HCG increased testosterone levels in both groups of rats. HCG stimulates androgen synthesis in the interstitial cells of the testes. It is suggested that the decrease in the secretion of testosterone produced by DEHP might lead to decreased testis weight and fertility.

PCB and reproduction

Orberg, J. (1978). Effects of pure chlorobiphenyls (2,4',5-trichlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl) on the post-natal growth in mice. *Acta pharmac. tox.* **42,** 275.

Orberg J. (1979). Effects of pure chlorobiphenyls (2,4',5-trichlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl) on the reproductive capacity in female mice. *Acta pharmac. tox.* **42,** 323.

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants which are likely to enter food chains. They have deleterious effects on reproduction in many animals (Barsotti *et al. Fd Cosmet. Toxicol.* 1976, **14,** 99; *Cited in F.C.T.* 1978 **16,** 289) and have been shown to be transferred from human mothers to foetuses and infants (Masuda *et al. Fd Cosmet. Toxicol.* 1978, **16,** 543). Their effects upon reproductive capacity and post-natal growth and development are therefore of great interest.

In the first experiment described, female mice were given 0·05 mg 2,4',5-trichlorobiphenyl (TCB) or 0·05 mg 2,2',4,4',5,5'-hexachlorobiphenyl (HCB) from day 5 of gestation until day 22 after parturition and their offspring were studied. At birth, HCB-treated males were significantly heavier than either TCB-treated males or controls, and this difference was sustained until day 35 after birth. Females showed no weight difference until day 21, when HCB-treated females were heavier than controls and TCB-treated females. By day 35, both groups of treated females were significantly heavier than controls.

The chlorobiphenyls affected the growth rates of the offspring. From day 21 to 35 the relative growth rates of both groups of treated males were significantly higher than those of control males. [The author defined relative growth rate as the gain in body weight during the period, expressed as a percentage of the body weight in the middle of the period, divided by the length of the period (%/day).] During this period the absolute growth rate (bodyweight gain/day) of HCB-treated males was also significantly greater than that of control males. Both absolute and relative growth rates were greater in TCB-treated females than in HCB-treated or control females from day 21 to 35. From day 5 to 20 after birth in both males and females there were no significant differences in growth rates between the three groups. Further studies would be necessary to discover the physiological basis for the stimulation of growth rate by the two PCBs.

The second experiment describes the effects on female mice of 0·05 or 0·5 mg HCB or TCB daily from day 1 to 6 of pregnancy. In both groups given the higher dose, frequencies of implanted ova were lower than in controls but at the lower dose no difference in implantation frequency appeared. The livers of mice given 0·5 mg HCB or 0·5 mg TCB daily contained higher concentrations of cytochrome *P*-450 on day 4 than those of animals taking the lower doses and of controls. There was no significant difference between animals given the same dose of HCB or TCB either in implantation frequency or in hepatic cytochrome *P*-450 levels. However, adipose tissue of HCB-treated mice contained a higher residual concentration than that of TCB-treated animals. No evidence emerged that biphenyls of high chlorine content have any more pronounced effect upon reproductive function than those of lower chlorine content.

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

More about TRIS

Blum, A., Gold, M. D., Ames, B. N., Kenyon, C., Jones, F. R., Hett, E. A., Dougherty, R. C., Horning, E. C., Dzidic, I., Carroll, D. I., Stillwell, R. N. & Thenot, J.-P. (1978). Children absorb tris-BP flame retardant from sleepwear: urine contains the mutagenic metabolite, 2,3-dibromopropanol. *Science, N.Y.* **201,** 1020.

Tris-(2,3-dibromopropyl) phosphate (TRIS) has been shown to have genetic effects upon eukaryotic cells as well as upon bacteria (*Cited in F.C.T.* 1979, **17,** 98) and to produce skin tumours and stomach papillomas after application to the skin of mice (*ibid* 1979, **17,** 688). The migration of TRIS from fabrics during laundering, and its variable degree of percutaneous absorption, has been demonstrated (*ibid* 1977, **15,** 257; *ibid* 1980, **18,** 207). In the study cited above the absorption by children of TRIS from treated sleepwear was estimated by determining urinary levels of a TRIS metabolite, 2,3-dibromopropanol (DBP), which is also mutagenic in the Ames test (Blum & Ames, *Science, N.Y.* 1977, **195,** 17). Urine from seven out of eight children wearing well-washed, TRIS-treated sleepwear contained about 0·5 ng DBP/ml, and that from an eighth child contained 5 ng DBP/ml. Traces of DBP were found in the urine of another child who was reported to have stopped wearing TRIS-treated sleepwear 6 months earlier, but none of the TRIS metabolite was detected in the urine of a child and an adult who had never worn sleepwear treated with this flame retardant.

A 7-year-old child, who had been wearing sleepwear that may have been treated with TRIS, wore new, TRIS-treated pyjamas for 5 nights and then untreated sleepwear for the subsequent 5 nights. Morning urine samples were taken throughout the study. Before wearing the new, TRIS-treated pyjamas the child's urine contained 0·4 ng DBP/ml. Samples taken after she had slept in the treated pyjamas contained up to 29 ng DBP/ml, and urine taken on the subsequent 5 days, when the child wore untreated sleepwear, contained DBP at levels of between 6 and 14 ng/ml.

The amount of TRIS metabolite absorbed is likely to be substantially higher than is indicated by the level of DBP in the urine since such analysis does not account for the amounts of TRIS or its metabolites that are stored in body tissues or excreted in faeces. Such storage in the body may explain why DBP excretion in the urine continued even after exposure to treated garments had stopped.

TEST PROCEDURES

Nitrosamines in test diets

Walker, E. A., Castegnaro, M. & Griciute, L. (1979). *N*-Nitrosamines in the diet of experimental animals. *Cancer Lett.* **6**, 175.

Last month we published an abstract of a report of the presence of low levels of volatile *N*-nitrosamines in laboratory-animal feeds (*Cited in F.C.T.* 1980, **18**, 208. In an earlier study Kann *et al.* (*Z. Krebsforsch.* 1977, **90**, 321) found similar levels in 46 samples of such feeds: 37 of the samples contained more than 1 ppb ($b = 10^9$) *N*-nitrosodimethylamine (NDMA) and 27 contained more than 1 ppb *N*-nitrosopyrrolidine (NPYR). The authors of both these studies emphasized the need to minimize nitrosamine levels in laboratory-animal diets. Now a group of IARC workers report similar findings, but suggest that these low levels of contamination of feeds with compounds known to be animal carcinogens may be put to good experimental use.

Volatile *N*-nitrosamines were found to be common contaminants in 87 samples of laboratory-animal feeds. The most prevalent compound was NDMA, which was present in 91% of the samples at concentrations of up to 54 ppb, although in the majority (75%) of the samples its concentration was only 1–10 ppb. Other *N*-nitrosamines were also detected, generally at levels of less than 10 ppb. *N*-Nitrosodiethylamine was present in 51% of samples, *N*-nitrosopiperidine in 19%, NPYR in 16%, *N*-nitrosodipropylamine in 15% and *N*-nitrosodibutylamine in 9%.

The authors consider that the volatile *N*-nitrosamines present in laboratory-animal feeds might contribute to the occurrence of spontaneous tumours in control animals. The incidence of these spontaneous tumours differs even among genetically pure strains of animals bred in different animal houses (Young & Hallowes in *Pathology of Tumours in Laboratory Animals*, p. 31, IARC, Lyon, 1973). It is suggested that an international network of laboratories should be set up in which the levels of volatile *N*-nitrosamines in feeds could be determined and compared with the incidence of tumours in control animals. Such collaboration between laboratories would provide an economical method of acquiring data on the effects of very low doses of *N*-nitrosamines in experimental animals.

EXPERIMENTAL PATHOLOGY

Mapping organophosphate-induced axonal degeneration

Bouldin, T. W. & Cavanagh, J. B. (1979). Organophosphorous neuropathy. I. A teased-fiber study of the spatio-temporal spread of axonal degeneration. *Am. J. Path.* **94**, 241.

Bouldin, T. W. & Cavanagh, J. B. (1979). Organophosphorous neuropathy. II. A fine-structural study of the early stages of axonal degeneration. *Am. J. Path.* **94**, 253.

Some organophosphorus compounds have delayed neurotoxic effects in certain species, including man, causing irreparable ataxia and paralysis. About 8–14 days after exposure the distal portions of the long axons of the central and peripheral nerves begin to degenerate, and the first biochemical step in the production of the lesions is the inhibition of a nervous system enzyme, neurotoxic esterase (Johnson, *Arch. Tox.* 1975, **34**, 259). In the studies cited above, the spatial–temporal spread of axonal degeneration in organophosphorus neuropathy was investigated, and the authors have concluded that such neuropathy does not involve a dying-back pattern of degeneration from the terminus of the axon, as was generally thought.

Young adult cats were given a single ip injection of 40 mg di-isopropyl fluorophosphate/kg and were killed 14, 18, 20, 21 or 28 days later by intracardiac infusion with aldehydes. Clinical signs of delayed neurotoxicity appeared 16–18 days after dosing. A histological study of the central and peripheral nervous systems revealed the axonal degeneration at the distal ends of the long nerves that is typical of a dying-back neuropathy. However light-microscopic examination of single nerve fibres teased from all levels of the left recurrent laryngeal nerve (LRLN) showed that although degeneration was limited to the distal axons in the vast majority of fibres, it did not begin at the axon terminus and progress back along

the axon. Instead it was initially localized at a focal point, usually a mid-internodal point, and subsequently spread to involve the entire distal axon in morphological stages identical to those of Wallerian degeneration.

Although no clinical signs of neurotoxicity and no histological signs of axonal degeneration of the LRLN were evident in the cat killed after 14 days, focal nerve fibre varicosities and paranodal demyelination were observed in teased LRLN fibres. These varicosities gave the fibres a beaded appearance and the number of beaded fibres was greater in animals showing clinical signs of ataxia.

The ultrastructure of the varicosities is described in the second paper cited above. Two cats were dosed with 40 mg di-isopropyl fluorophosphate/kg and were killed 18 and 20 days later. Sections of four LRLN nerve fibres in progressive stages of axonal degeneration were selected for examination by electron microscopy. The varicosities were shown to be associated with intra-axonal and/or intramyelinic vacuoles. In all four fibres there were accumulations of axonal agranular reticulum, but these were associated with less than half of the varicosities.

From these two studies the authors conclude that the organophosphate induces a focal, non-terminal degeneration of the axon which produces a 'chemical transection' of the nerve fibre. This 'chemical transection' then precipitates Wallerian degeneration of the distal axon. Further study is required to establish the pathogenesis of the two types of vacuole and their interrelationship and to confirm the apparent evolvement of the varicosities into the focal areas of axonal degeneration.

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LETTER TO THE EDITOR

NITROSAMINES IN DISHWASHING COMPOUNDS

Sir,—Our laboratory is actively engaged in the determination of volatile nitrosamines in food products. We recently observed a sample-contamination problem that we believe should be brought to the attention of other investigators determining nitrosamines. *N*-Nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA) and *N*-nitrosodiethylamine (NDEA) were occasionally detected, using the Thermal Energy Analyzer (TEA), in control/blank samples that should not have had TEA-responsive peaks. A quick investigation revealed that the source of the nitrosamines was the dishwashing compound used to clean the glassware. A limited survey of some commercially available dishwashing compounds showed that a few samples had detectable levels of nitrosamines ranging from 1 to 11 ppb NDMA, from 2 to 5 ppb NMEA, and from 1 to 8 ppb NDEA. Mass-spectrometric confirmation of a single sample containing both NDMA and NDEA proved positive for both nitrosamines. This finding takes on added importance because of the recent disclosure by NIOSH that testing of the detergent and surfactant industry for nitrosamines will soon take place (*Chemical & Engineering News* 1980, **58** (13), 23). Therefore, necessary precautions should be taken and dishwashing compounds should be analysed to prevent nitrosamine artefacts in analytical procedures.

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MEETING ANNOUNCEMENTS

MICROBIOLOGY AND ENGINEERING OF STERILIZATION PROCESSES

The Parenteral Drug Association are sponsoring a 3-day intensive course entitled "Microbiology and Engineering of Sterilization Processes" for those involved in the research, development and manufacture of sterilized products. Lectures and activities on the first day come under the heading "Microbiological Basis of Sterilization Analysis" while the subject of the second day is "Analysis of Heating Data, Calculating Processes" and the third day is devoted to Designing, Validating and Monitoring Sterilization Processes. The course will be conducted by Dr. Irving J. Pflug, Professor of Public Health and Food Sciences at the University of Minnesota.

The course will be held at the Cavendish Hotel, Jermyn St., London and the dates of the two alternative sessions are 10, 11 and 12 September or 16, 17 and 18 September 1980. Further information may be obtained from Gerry Prout, Parenteral Drug Association, Inc., 22 Ermin Close, Baydon, Marlborough, Wiltshire (telephone no. Swindon (0793) 24411, ext. 32).

FOOD—TIME FOR CHANGES?

"Composition, Safety and Processing of Food—the Desirability of Change" is the title of the Annual Symposium of the Institute of Food Science and Technology to be held at St. John's College, Cambridge on 3–5 September 1980. Further details are available from Mr. A. Hobson-Frohock, ARC Food Research Institute, Colney Lane, Norwich NR4 7UA.

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CORRIGENDA

Volume 18 (1980)

p. 55, The date on which this paper was received was 12 May 1979 not 12 May 1970

Volume 17 (1979)

The following paper previously appeared on p. 591 of volume 17. It is reprinted here in full with corrections in order to clarify any confusion caused by a printing error in the original discussion.

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BIOLOGICAL EFFECTS OF ALKALI-TREATED SOYA PROTEIN AND LACTALBUMIN IN THE RAT AND MOUSE*

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(Received 9 April 1979)

Abstract—Diets containing 20% alkali-treated soya protein and providing 1370–2630 ppm dietary lysinoalanine produced nephrocytomegaly in the *pars recta* of the proximal tubule in male Sprague-Dawley and Wistar rats. The frequency of abnormally large *pars recta* nuclei exceeded 33% in rats fed alkali-treated soya protein providing dietary lysinoalanine levels of 2080 or 2630 ppm. There were no quantitative or qualitative differences between the renal responses of Sprague-Dawley and Wistar rats fed the same diets. Swiss-Webster mice fed alkali-treated soya protein had slightly more than twice as many enlarged *pars recta* nuclei as did the control group, but the shift in size distribution was not significant at the 5% level. Sprague-Dawley rats developed only minimal karyomegaly on a diet of 20% alkali-treated lactalbumin, despite a dietary lysinoalanine level of 4970 ppm. Diets containing 8 or 10% untreated lactalbumin in addition to 12% alkali-treated soya protein or 10% alkali-treated lactalbumin did not induce nephrocytomegaly or karyomegaly, despite providing dietary lysinoalanine levels of 1753 and 2490 ppm, respectively. Sprague-Dawley and Wistar rats fed 20% alkali-treated soya protein showed elevated plasma levels of glutamic-pyruvic transaminase (GPT) and blood urea nitrogen (BUN), and had a significantly lower plasma protein content than control animals after 8 or 12 wk on the diet. Sprague-Dawley rats fed 20% alkali-treated lactalbumin had elevated GPT levels, but BUN and total plasma protein were normal; replacement of half the protein content of the diet with non-nutritive cellulose caused elevation of GPT, with no change in the BUN or plasma protein values. In rats fed 20% alkali-treated soya protein, approximately 33% of the lysinoalanine ingested daily was found in the faeces and 1·2% in the urine. No lysinoalanine was detected in liver or blood, but a quantity equivalent to about 0·6% of the daily intake was found in the kidneys. Higher urinary lysinoalanine excretion (2–7% of the intake) was found in animals fed alkali-treated lactalbumin.

INTRODUCTION

The fact that ingestion of alkali-treated proteins induced by alkali-treated protein (Gould & MacGregor, 1977); there are marked species variations in sensitivity to lysinoalanine-induced nephrocytomegaly (de Groot, Slump, Feron & van Beek, 1976) and the Woodard, Short, Alvarez & Reyniers, 1975), has raised concerns about the presence of lysinoalanine in human foods (Gould & MacGregor, 1977). Many commercial and common cooked foods contain lysinoalanine, in some cases at levels near, or even well above, those which produce nephrocytomegaly in rats (Sternberg & Kim, 1977; Sternberg, Kim & Schwende, 1975). However, a number of variables strongly modify the development in the rat of lesions induced by alkali-treated protein (Gould & MacGregor, 1977); there are marked species variations in sensitivity to lysinoalanine-induced nephrocytomegaly (de Groot, Slump, Feron & van Beek, 1976) and the relationship of the nephrocytomegaly to its possible health consequences is still unclear (Gould & MacGregor, 1977).

This report describes the results of experiments designed to investigate the biological effects of alkali-treated soya protein and alkali-treated lactalbumin in Wistar and Sprague-Dawley rats and in Swiss-Webster mice.

EXPERIMENTAL

Alkali treatment of soya protein and lactalbumin. Three batches of alkali-treated soya protein isolate, designated $ATSI_1$, $ATSI_2$, $ATSI_3$, were prepared at different times, using the conditions described by Woodard & Short (1973). The 0·1 N-NaOH solution was preheated to 60 ± 1°C. The soya protein (Promine D, Central Soya Chemical Division, Chicago, IL) was dispersed slowly with vigorous stirring to prevent formation of clumps. Water lost by evaporation during the 8-hr treatment period was replaced. The initial pH after suspending the protein was 12·1, and after the 8-hr treatment period the pH was 10·8. After

*Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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treatment, the pH was adjusted to 4·6 with 6 N-HCl until it remained stable for more than 15 min. The suspension was left to settle overnight in a coldroom at 2°C. The supernatant was siphoned off, the precipitate was remixed with ten volumes of distilled water, and again left to settle overnight at 2°C. After a total of three such washings with distilled water, the precipitate was drained overnight in perforated baskets lined with cheesecloth and was subsequently spread in trays, frozen, and freeze-dried. The protein recovery was 82–85%. The lysinoalanine contents were: $ATSI_1$, 0·68%; $ATSI_2$, 0·98%; $ATSI_3$, 1·27%.

A single batch of alkali-treated lactalbumin was prepared by dispersing 3·2 kg lactalbumin (alcohol extracted, Nutritional Biochemicals Corp., Cleveland, OH) in 93 litres of 0·1 N-NaOH at 60°C for a treatment time of 80 min. The initial pH after suspending the protein was 12·2, the pH after the alkaline treatment was 11·6. After treatment, the protein was precipitated by adding 2 N-HCl to achieve a pH of 3·9. The supernatant was discarded, the precipitate was washed once with 80 litres of distilled water and freeze-dried. The protein recovery was 89%. The lysinoalanine content of the final dried protein was 2·5%.

Protein hydrolysis and amino acid analysis. Protein hydrolysis and amino acid analyses were carried out as described previously (Karayiannis, MacGregor & Bjeldanes, 1979).

Diets. The diet used was similar to that used by Woodard (1969). It was composed of 20% protein, 64·4% sucrose, 10% corn oil, 5% salt mix, 0·35% vitamin mix and 0·3% DL-methionine. The salt mix was a modification of that described by Hegsted, Mills, Elvehjem & Hart (1941). It contained (in g/100 g salt mixture), K_2HPO_4, 32·2; $CaCO_3$, 30; NaCl, 16·7; $MgSO_4 \cdot H_2O$, 10·2; $CaHPO_4$, 7·5; $Fe(C_6H_5O_7)_2 \cdot 6H_2O$, 2·7; $MnSO_4 \cdot H_2O$, 0·5; KI, 0·08; $CuSO_4 \cdot 5H_2O$, 0·03; $ZnCl_2$, 0·025. The vitamin mix was similar to the commercial vitamin diet fortification mixture—1369 of ICN—Nutritional Biochemicals Corp., Cleveland, OH. It supplied (in mg/100 g diet) choline chloride, 165; ascorbic acid, 99; thiamine-HCl, 2·2; pyridoxine-HCl, 2·2; riboflavin, 2·2; menadione, 4·95; Ca-pantothenate, 6·6; nicotinic acid, 9·9; *p*-aminobenzoic acid, 11; inositol, 11; α-tocopherol, 12·1; folic acid, 0·20; biotin, 0·044; vitamin B_{12}, 0·003; and in IU/100 g diet: vitamin A-acetate, 2500; vitamin D_3, 600. The diets in which lactalbumin was the protein source were supplemented with 0·4% L-arginine HCl. In diets in which two different proteins were present, the total protein content of the diet was always 20%.

Animals. Male weanling rats (about 21 days old, 50–65 g) of two different strains, Sprague-Dawley and Wistar, and male weanling Swiss-Webster mice (10–11 g) were used in the 8-wk feeding studies. Animals were obtained from Simonsen Laboratories, Inc., Gilroy, CA. Food and tap-water were supplied *ad lib.* except for the pair-fed groups. Food consumption and body weight of the groups fed *ad lib.* were recorded weekly. Animals were examined daily for gross clinical abnormalities. Individually caged, pair-fed control animals were given a daily food allotment equal to the quantity consumed on the previous day by a paired rat receiving the experimental diet.

Tissue and sample collection. At necropsy, animals were anaesthetized with ether (mice and lactalbumin-fed rats) or 70 mg phenobarbital/kg body weight (groups fed soya protein). The chest cavity was opened and blood was drawn from the right ventricle for subsequent blood cell counts and plasma enzyme analyses. EDTA (3 mg/ml blood) was used as the anticoagulant for blood cell counts and potassium oxalate (2 mg/ml blood) for enzyme analyses. Animals were examined for signs of gross pathological change. Kidneys were removed and weighed. The left kidney was cut in half transversely (across the longer axis), and the right, longitudinally (parallel to the longer axis), such that two symmetrical pieces resulted. One half of each kidney was fixed in 10% buffered formalin and the other half was frozen for subsequent chemical analyses. The livers of the animals fed $ATSI_3$ and controls were immediately frozen between two slabs of dry ice for subsequent determination of *N*-demethylase activity.

In addition to the blood samples obtained at necropsy, blood samples were taken from the ventral tail artery of five Sprague-Dawley and five Wistar rats fed $ATSI_3$, and from the corresponding control groups, (Table 1, groups 4, 6, 8, 10) after 2, 11, 23 and 44 days on the test diets. These samples were immediately drawn from the hub of the needle used for the arterial puncture into a 40 μl capillary tube and were diluted into 20 ml of Isoton counting fluid (Coulter Electronics, Inc., Hialeah, FL). No anticoagulant was used. Red and white blood cell counts were performed as described below.

Urine and faeces were collected separately in metabolism cages. Animals were housed individually. A small crystal of thymol was placed in each urine collecting vial to inhibit bacterial growth. Samples were frozen immediately after collection. During the first 12 hr of urine collection no food was given, to avoid contamination of urine and faeces with lysinoalanine from the diet. Three rats from the $ATSI_3$-fed group were returned to a lysinoalanine-free control diet after this 12-hr period, and urine and faeces were then collected for a five-day period.

Histological measurements. The right kidney of each animal was sectioned as follows. The kidney was placed with the cut surface up and a razor blade was directed perpendicular to the cut surface parallel to the long axis of the kidney at a level just below the outer medullary stripe. The resulting corticomedullary wedge, after processing, was sectioned parallel to the plane of the last cut surface (frontal plane).

Kidney wedges were processed through ethanol, xylene and paraffin, and embedded in paraffin blocks (Luna, 1968). Frontal sections 5 μm thick were cut, mounted on microscope slides, dried overnight at 60°C, and stained with haematoxylin and eosin.

The size distribution of nuclei in the affected regions of the outer medullary stripe of the kidney was quantified as follows. The slides containing stained kidney sections were coded and randomized to avoid any subjective bias. The observer was not aware of the identity of the slides examined. The slide was scanned under a microscope which permitted either direct visual examination or projection of the image onto an 18 × 23 cm television monitor at a magnification of 1430×. The magnification was cali-

brated using a stage microscopic scale. One centimetre on the TV screen corresponded to 7 μm on the slide, permitting the diameter of nuclei to be measured to within 0·5 μm. Observation was started in the area of the outer medullary stripe judged to have the highest density of enlarged nuclei. Once measurements were begun, a predetermined and consistent scanning pattern was used. All *pars recta* nuclei in the projected area were measured and recorded. A total of eight screens, containing a total of 280–450 nuclei, were scored for each animal. Nuclei contained in collecting ducts, blood vessels, etc. were not included. Nuclei were classified into classes of 5·6–6·9, 7·0–8·3, 8·4–9·7, 9·8–11·1, 11·2–12·5 and 12·6–14·0 μm in diameter. In elongated nuclei the major and minor axes were averaged (Elias, Hennig & Schwartz, 1971). This method of scoring karyomegaly correlated well with subjective ranking of the severity of karyomegaly when extensive karyomegaly was present and, in addition, permitted the detection of relatively small shifts in the size distribution which were impossible to detect by subjectively ranking the degree of karyomegaly or nephrocytomegaly.

Blood cell counts, liver N-demethylase activity and plasma chemistries. Red and white blood cell counts were performed using a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Commercially available cell diluent (Isoton), cell-lysing agent (Zap-o-globin) and blood cell standards were obtained from Coulter Electronics, Inc.

For measurement of liver *N*-demethylase activity, frozen liver samples were chopped without thawing. Approximately 5 g liver and 20 ml ice-cold isotonic KCl (buffered with 0·01 M-phosphate buffer, pH 7·4) were weighed into a tared homogenizer tube and the liver was homogenized with a motor-driven teflon pestle and centrifuged at 10,800 g for 30 min. *N*-Demethylase activity in the supernatant was determined by measuring the rate of formaldehyde release from aminopyrine (Dalton & Di Salvo, 1972).

Blood samples were centrifuged at 8000 g for 30 min at 2°C immediately after collection. Plasma was pipetted into 3 ml plastic covered cups and frozen until analysis. All blood chemistries were performed on a Technicon Autoanalyzer II, using standard methods described in detail in the Technicon Manual (Technicon Instruments Corp., Tarrytown, N.Y.). The date and reference number of the technical bulletins describing each assay are: glutamic–pyruvic transaminase, AAII-22, April 1971; glutamic–oxaloacetic transaminase, AAII-10, June 1971; blood urea nitrogen, AAII-01, December 1970; total protein, AAII-14, October 1970; albumin, AAII-15, October 1970. Ornithine carbamoyl transferase was determined by the method of Strandjord & Clayson (1966).

Protein efficiency ratios (PER's) and digestibility tests were carried out using the standard AOAC method (Association of Official Analytical Chemists, 1975), using five weanling Sprague-Dawley rats per group. The diets consisted of 10% protein (calculated as $N \times 6{\cdot}25$), 20% corn starch, 8% corn oil, 5% USP salt mixture XIV supplemented with 0·055% $ZnSO_4 \cdot 7H_2O$ and 0·0023% $CoCl_2 \cdot 6H_2O$, 3% cellulose, 2% vitamin mix (see above) and dextrose to make 100%. All diets had an equal quantity of nitrogen, fat, fibre and water. The diets containing soya protein were supplemented with 0·3% DL-methionine. The untreated diet containing lactalbumin was supplemented with 0·4% L-arginine–HCl- and the alkali-treated lactalbumin diets with 0·49% L-arginine–HCl. Reference casein, approved by the Animal Nutrition Research Council supplied by Nutritional Biochemicals, Inc. (Cleveland, OH) was also tested.

RESULTS

Nephrocytomegaly, growth and feed utilization

The occurrence of nephrocytomegaly and growth effects observed in the groups of rats and mice fed the various diets containing alkali-treated proteins are summarized in Table 1. The diets containing 20% alkali-treated soya protein, and providing 1370 ppm to 2630 ppm dietary lysinoalanine, consistently produced nephrocytomegaly in rats. The histological appearance of the lesions was in accordance with that observed by Woodard (1969) and Woodard & Alvarez (1967) in Sprague-Dawley rats fed alkali-treated soya protein. Representative sections of the renal outer medullary stripe of Sprague-Dawley and Wistar rats fed alkali-treated or untreated soya protein, and of Sprague-Dawley rats fed alkali-treated lactalbumin are shown in Fig. 1.

The size distributions of nuclei in the affected regions of the outer medullary stripe are given in Fig. 2. In control rats of both strains, the frequency of nuclei exceeding 8·4 μm in diameter was less than 0·5%, while one-third or more of the nuclei exceeded this dimension in rats fed $ATSI_3$ or $ATSI_2$. There were no quantitative or qualitative differences in the responses of Wistar and Sprague-Dawley rats fed the same diet (Figs 1 and 2, Table 1).

Despite providing a dietary lysinoalanine level of 4970 ppm, the alkali-treated lactalbumin induced only mild karyomegaly when fed at 20% in the diet (Fig. 1 and 2). This protein was more mildly treated (see methods) and permitted greater growth than an equivalent amount of the alkali-treated soya protein. The 10% alkali-treated lactalbumin–10% untreated lactalbumin diet permitted normal growth and did not cause nephrocytomegaly (Table 1, Fig. 2), even though the dietary lysinoalanine level (2490 ppm) was roughly equivalent to that provided by the 20% $ATSI_3$ (2630 ppm) or the 20% $ATSI_2$ (2080 ppm) diet. Further, diet no. 19 (12% $ATSI_3$, 8% untreated lactalbumin) did not produce nephrocytomegaly, even though the dietary lysinoalanine (1753 ppm) exceeded that of the 20% $ATSI_1$ diet (1370 ppm).

Although the animals on the alkali-treated soya protein diets ate less and had a lower feed efficiency than did those on untreated protein, the lower food intake and lower protein availability did not, in themselves, induce nephrocytomegaly. Pair-fed groups (Table 1, groups 12, 13) and groups with the protein partly replaced by Alphacel (Table 1, groups 17, 18) did not develop nephrocytomegaly, even when the protein restriction reduced their growth below that observed with the alkali-treated protein. While this type of control is adequate for the overall reduction in protein availability which is known to result from alkali treatment (de Groot & Slump, 1969), it must be noted that alkali treatment could modify the balance of available amino acids by specifically reducing the

Table 1. *Nephrocytomegaly, growth and feed efficiency in Sprague-Dawley and Wistar rats and in Swiss-Webster mice fed diets containing alkali-treated proteins for 8 wk*

Group	No. in group, strain, species	Dietary protein*	Dietary lysinoalanine (ppm)	Final body weight (g)†	Average 8-wk feed efficiency (g gain/g feed consumed)	Nephrocytomegaly‡
1	5, SD, Rat	UTSI	0	360 ± 11	0·37	0 (5)
2	5, SD, Rat	$ATSI_1$	1370	293 ± 12	0·35	+ + (5)
3	5, SD, Rat	$ATSI_2$	2080	210 ± 4	0·25	+ + + (5)
4	10, SD, Rat	UTSI	0	355 ± 8	0·40	0 (6)
5	10, SD, Rat	$ATSI_3$	2630	229 ± 8	0·31	+ + + (7)
6	5, SD, Rat	UTSI§	0	406 ± 14	—	0
7	5, SD, Rat	$ATSI_3$§	2630	334 ± 14	—	+ + +
8	10, WIS, Rat	UTSI	0	364 ± 5	0·42	0 (7)
9	10, WIS, Rat	$ATSI_3$	2630	225 ± 9	0·31	+ + + (7)
10	5, WIS, Rat	UTSI§	0	439 ± 6	—	0
11	5, WIS, Rat	$ATSI_3$§	2630	309 ± 25	—	+ + +
12	5, SD, Rat	UTSI‖	0	319 ± 16	0·37	0 (5)
13	5, SD, Rat	$ATSI_3$	2630	270 ± 17	0·29	+ + + (5)
14	7, SD, Rat	UTL	0	371 ± 8	0·39	0 (7)
15	7, SD, Rat	ATL	4970	294 ± 9	0·35	+ (7)
16	7, SD, Rat	UTL + ATL (1:1)	2490	339 ± 8	0·33	0
17	7, SD, Rat	UTL + Alphacel (1:1)	0	253 ± 10	0·26	0 (5)
18	5, SD, Rat	UTL + Alphacel (2:3)¶	0	210 ± 13	0·26	0 (5)
19	5, SD, Rat	UTL + $ATSI_3$ (2:3)¶	1753	283 ± 16	0·40	0 (5)
20	7, SW, Mouse	UTSI	0	34 ± 1·2	—	0 (7)
21	7, SW, Mouse	$ATSI_3$	2630	30 ± 0·5	—	0 (7)
22	7, SW, Mouse	UTL + $ATSI_3$ (2:3)	1580	34 ± 1·3	—	0 (7)

UTSI = Untreated soya protein isolate
$ATSI_1$ = Alkali-treated soya protein isolate (containing 0·68% lysinoalanine)
$ATSI_2$ = Alkali-treated soya protein isolate (containing 0·98% lysinoalanine)
$ATSI_3$ = Alkali-treated soya protein isolate (containing 1·27% lysinoalanine)
SD = Sprague-Dawley WIS = Wistar SW = Swiss-Webster

*See text for detailed description. Diets contained 20% of the indicated protein or mixture (in the ratio given in parentheses). For example, the diet for group 18 contained 8% untreated lactalbumin and 12% Alphacel, by weight.

†Mean ± SEM.

‡Summary of results from Fig. 2. The symbol 0 indicates no nephrocytomegaly; + indicates an increase in nuclear size which is significant at the $P < 0·05$ level; + + indicates that at least 10% of nuclei in affected regions exceeded a diameter of 8·4 μm; + + + indicates that at least 25% of nuclei in affected regions exceeded a diameter of 8·4 μm. The number in parentheses is the number of animals from each group upon which the frequency distribution of *pars recta* nuclei in Fig. 2 was based. Groups not shown in Fig. 2 (6, 7, 10, 11, 16) were examined for nephrocytomegaly but complete frequency distributions were not measured for those groups.

§These groups were fed the experimental diets for 12 wk.

‖Pair-fed with group 13.

¶Pair-fed.

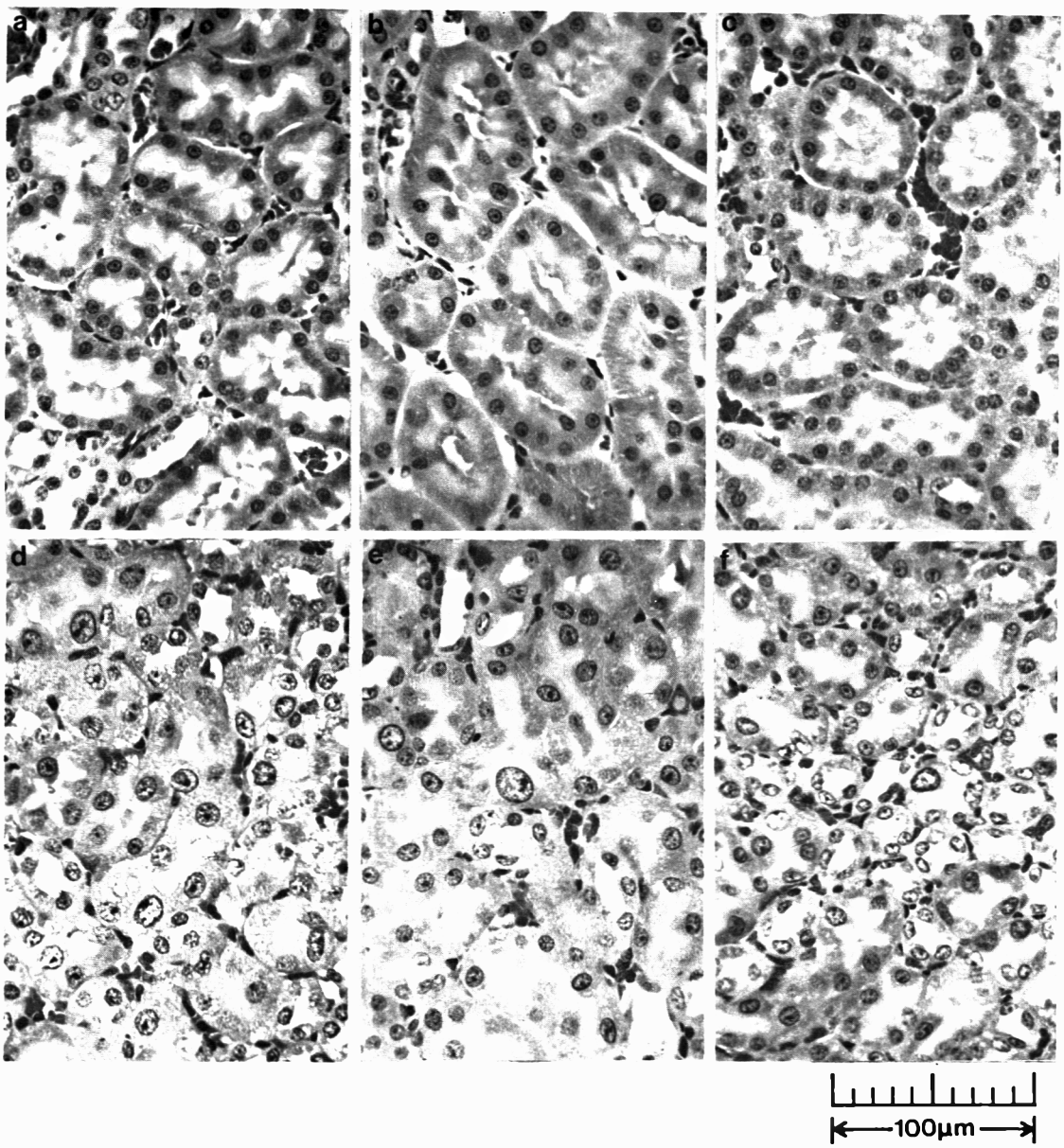

Fig. 1. Histological appearance of the renal outer medullary stripe of rats fed untreated or alkali-treated soya protein or lactalbumin for 8 wk (a) 20% untreated soya protein. Sprague-Dawley strain (b) 20% untreated soya protein. Wistar strain (c) 20% untreated lactalbumin. Sprague-Dawley strain (d) 20% alkali-treated soya protein ($ATSI_1$). Sprague-Dawley strain (e) 20% alkali-treated soya protein ($ATSI_3$). Wistar strain (f) 20% alkali-treated lactalbumin (ATL). Sprague-Dawley strain.

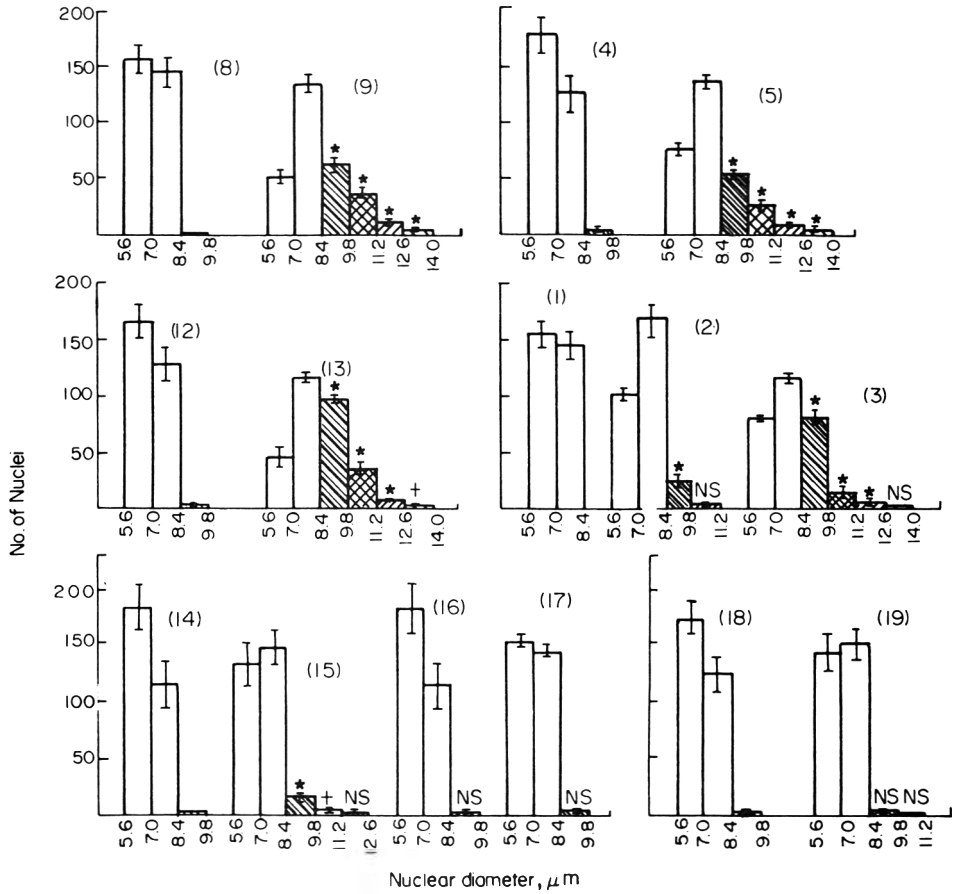

Fig. 2. Size distribution of *pars recta* nuclei in 5 μm kidney sections from rats fed 20% untreated or alkali-treated protein for 8 wk. Data are normalized to 300 nuclei/animal. The histograms correspond to the group numbers given in brackets. See Table 1 for details of each group. Bars indicate $\pm$ one standard error. Significant increases in the number of nuclei in size groups above 8·4 μm, compared with the same size group in the untreated controls, are indicated; $*P < 0{\cdot}01$; $\dagger P < 0{\cdot}05$ (nonparametric two-tailed comparison by rank-sum test (Wine, 1964)).

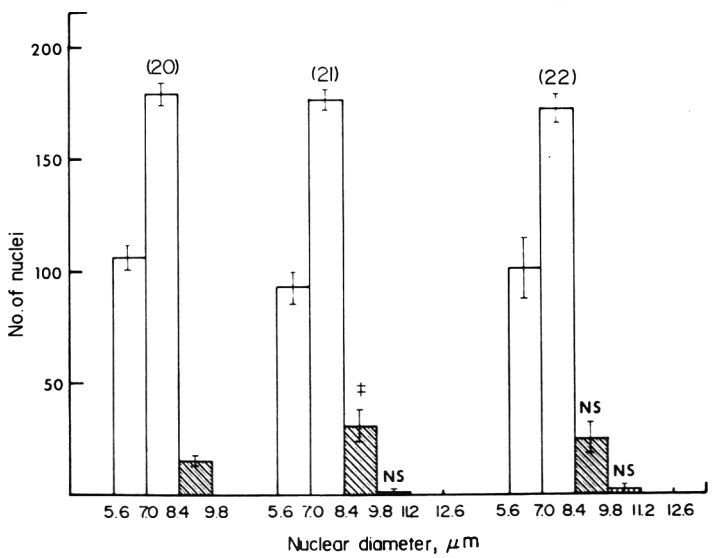

Fig. 3. Size distribution of *pars recta* nuclei in mice fed untreated soya protein, alkali-treated soya protein ($ATSI_3$), or a mixture of $ATSI_3$ with untreated lactalbumin for 8 wk. See Table 1 for details of the group numbers given in brackets. Bars represent one standard error. The result marked with a double dagger was not significantly different from control at $P < 0{\cdot}05$ by either nonparametric rank-sum test or by Student's *t*-test; however, $P < 0{\cdot}05$ in a one-tailed rank-sum test and $P \sim 0{\cdot}075$ by Student's two-tailed *t*-test.

Table 2. *Protein efficiency ratios and digestibility data for alkali-treated lactalbumin and soya isolate*

Dietary source of protein	Final body weight† (g)	Total feed consumption (g)	Protein efficiency ratio*		Digestibility* (%)	
			Actual	Adjusted	Diet	Nitrogen
ANRC protein	212 ± 15^{A}	430 ± 37^{A}	3·65 ± 0·06^{AB}	2·50	95	94
Lactalbumin	199 ± 13^{A}	375 ± 30^{A}	3·83 ± 0·05^{A}	2·62	95	90
ATL‡	107 ± 4^{B}	216 ± 10^{B}	2·42 ± 0·10^{C}	1·66	95	81
Soya isolate	201 ± 11^{A}	420 ± 26^{A}	3·47 ± 0·09^{B}	2·38	94	90
$ATSI_3$	108 ± 2^{B}	275 ± 11^{B}	1·92 ± 0·07^{D}	1·32	94	82

ANRC protein = Reference casein, approved by the Animal Nutrition Research Council
ATL = Alkali-treated lactalbumin $ATSI_3$ = Alkali-treated soya protein isolate containing 1·27% lysinoalanine)
Values were compared using Duncan's multiple comparison test. Means without a superscript letter in common are significantly different, $P < 0·01$.
*The protein efficiency ratio assay was 28 days. Digestibility data was from day 6 through day 13 of the test, means ± SEM.
†Five male weanling rats per group, Sprague-Dawley strain, initial age 21 days, mean initial weight = 55 grams.
‡Entire group had diarrhoea beginning on day 13.

availability of particular amino acids more than others. This would be very difficult, if not impossible, to mimic in control diets that are not alkali-treated.

In mice, the 20% $ATSI_3$ diet resulted in significantly ($P < 0·01$) reduced these indices in both lactalbumin and soya isolate. The PER of lactalbumin was (Fig. 3). The observed incidence of nuclei with diameters exceeding 8·4 μm was more than doubled over the group fed untreated soya protein however, and the significance level was less than 0·05 in a one-tailed, but not in a two-tailed comparison using the rank-sum test (Wine, 1964). It is noteworthy that Feron, van Beek, Slump & Beems (1977) report observing nephrocytomegaly in Swiss mice fed high levels of free lysinoalanine.

The feed efficiency of the animals fed alkali-treated proteins was always lower than that of the control animals (Table 1). PER and digestibility determinations (Table 2) showed that alkali treatment significantly ($P < 0·01$) reduced these indices in both lactalbumin and soya isolate. The PER of lactalbumin was reduced by 37% and that of soya isolate by 45%. The nitrogen digestibility of the proteins was reduced by the alkali treatment, from an initial value of 90%, to 81% and 82% for lactalbumin and soya protein, respectively. Alkali treatment reduced the PER value considerably more than the digestibility. This is in agreement with the finding of de Groot & Slump (1969) who reported that decreased net protein utilization was the earlier and more pronounced finding in alkali-treated proteins. The animals fed the alkali-treated soya protein test diet used in the pair-feeding experiment (Table 1, groups 12 and 13) excreted almost twice as much faecal nitrogen as did animals on the corresponding untreated soya protein diet (Table 3). These pair-fed groups had uncorrected nitrogen digestibility values of 89% and 94%, respectively.

Approximately 30% of the test animals developed diarrhoea, which began on the second day on diet, and improved after 2–3 wk. Animals fed the diets containing Alphacel did not develop diarrhoea. Diarrhoea was more severe in the animals fed alkali-treated lactalbumin than in those fed alkali-treated soya protein. It persisted up to the third to fourth week on the diet and became less severe thereafter.

Approximately 30% of the animals fed alkali-treated soya protein, but not those fed alkali-treated lactalbumin, suffered loss of hair (alopecia). Alopecia developed in the third week and ranged from severe (three quarters of the body surface) in some animals, to patches of less than one-fifth of the body surface. After 6–7 wk on the diet all animals had recovered and the hair had grown back. The distribution pattern of diarrhoea and alopecia among the cages and the pattern of occurrence in experiments performed at different times made the possibility of an infectious agent unlikely. Diarrhoea in rats fed alkali-treated soya isolate in diets similar to those we used has been reported (Van Beek, Feron & de Groot, 1974). Hair-loss similar to that observed has been reported in rats fed lysine-deficient diets (Gershoff *et al.* 1958). The onset of the hair-loss reported was at the third week as it was in our experiments. None of the animals on the alkali-treated lactalbumin diet suffered any hair-loss. The alkali-treated lactalbumin was of better nutritional quality than alkali-treated soya protein having a 17% higher PER value than the treated soya protein. Amino acid determination (Table 4) showed that alkali-treated lactalbumin contained higher amounts of most of the essential amino acids than $ATSI_3$. It also contained almost twice as much lysinoalanine. The difference in arginine content was eliminated by supplementing lactalbumin with 0·4% L-arginine–HCl. It appears that deficiency or imbalance of certain amino acids in the alkali-treated soya protein was poorly tolerated during the period of most rapid growth and became less important later, as evidenced by the recovery from alopecia. Test animals had a poor appearance, but no deaths or other abnormalities were observed. Gross necropsy findings were essentially negative.

Clinical chemistry and haematology

Animals on the alkali-treated protein diets exhibited significantly elevated levels of plasma GPT and of blood urea nitrogen (BUN, Table 5). The elevation occurred in both the Sprague-Dawley and Wistar strains. In the animals fed alkali-treated lactalbumin, there were no statistically significant changes in the GPT or BUN levels. In a group of three animals

Table 3. *Urinary and faecal lysinoalanine excretion and faecal nitrogen, fat and ash excretion in Sprague-Dawley rats ingesting alkali-treated soya protein or alkali-treated lactalbumin**

Parameter	20% Soya isolate†	20% Alkali-treated soya isolate†	20% Alkali-treated lactalbumin	10% Lactalbumin 10% Alkali-treated lactalbumin
Dietary LAL (ppm)	0	2630	4970	2475
LAL intake (mg/day)	—	41 ± 3·3 (5)‡	72§	40§
Urinary LAL excretion‖ (unhydrolyzed, mg/day)	—	0·50 ± 0·12 (3)	2·7 ± 0·70 (4)	0·37 (2)
Urinary LAL excretion (individual samples, before/after acid hydrolysis, mg/day)	—	—	4·7/5·0; 2·2/3·2	0·79/1·1; 0·78/0·98
Faecal LAL excretion (hydrolyzed, mg/day)	—	14 ± 2·3 (5)	—	—
Faecal nitrogen excretion (mg/day)	29 ± 4 (5)	55 ± 4 (5)¶	—	—
Faecal fat excretion (mg/day)	63 ± 6 (5)	52 ± 5 (5)	—	—
Faecal ash (mg/day)	199 ± 21 (5)	187 ± 16 (5)	—	—

LAL = Lysinoalanine

*Urinary excretion data were obtained after 36–37 days on the diet specified. Rats were approximately 8 wk old at this time. Faecal excretion data were obtained from five pair-fed rats per group from samples collected during the 38th to 48th day on the diet.

†Pair-fed groups.

‡Average intake during the 10-day period of faeces collection.

§Average intake during the 7 days preceding urine collection, determined on a group of seven rats including those from which urine was collected.

‖Measured during a 12-hr period following removal of the diet.

¶ $P < 0·002$ compared with group fed untreated soya isolate.

which was fed $ATSI_3$ for 8 wk and then returned to laboratory chow at week 9 the BUN returned to the control value by week 12, but the GPT value was still significantly higher at the $P < 0·05$ level (data not shown). Total plasma protein values were significantly lower in the $ATSI_3$ fed rats after 8 wk on the diet (Table 5). Plasma albumin levels were also generally lower in the groups receiving alkali-treated protein (Table 5), but these values were not statistically significantly different from control values, implying that the plasma globulin values (which were not measured) were also lower in these rats.

Plasma glutamic–oxaloacetic transaminase activity (GOT) values were not elevated in animals fed alkali-

Table 4. *Amino acid composition of soya isolate and lactalbumin before and after alkali treatment with 0·1 N-NaOH at 60°C*

	Amino acid content (μmol/0·16 g N)			
	Soya isolate		Lactalbumin	
Amino acid	Untreated	0·1 N-NaOH 60°C, 8 hr ($ATSI_3$)	Untreated	0·1 N-NaOH 60°C, 1 hr 20 min
Asp	964	935	944	964
Thr	327	312	480	470
Ser	535	505	485	473
Glu	1474	1356	1226	1174
Pro	513	478	473	453
Gly	584	563	326	320
Ala	477	476	640	638
Val	436	447	536	547
Met	117	101	181	159
Cys	122	39	291	107
Ileu	390	389	446	443
Leu	660	662	1026	1075
Tyr	222	223	241	244
Phe	347	347	249	242
LAL	—	57	—	122
His	170	160	141	115
Lys	450	356	686	541
Arg	479	448	197	133

Table 5. *Blood chemistry of Wistar and Sprague-Dawley rats fed alkali-treated soya protein isolate or alkali-treated or untreated lactalbumin at a dietary level of 20%*

Group no. †	Dietary protein	Weeks on diet	No. of rats/group	GPT (mU/ml)	BUN (mg % N)	Total protein (g%)	Albumin (g%)
				Wistar rats			
4	UTSI	8	10	$11 \pm 0{\cdot}5^{ef}$	$14 \pm 1{\cdot}03^{cd}$	$6{\cdot}9 \pm 0{\cdot}10^{bcd}$‖	$2{\cdot}9 \pm 0{\cdot}11^{abc}$
5	$ATSI_3$	8	10	$^{***}29 \pm 2{\cdot}0^{a}$	$^{**}19 \pm 0{\cdot}98^{a}$	$^{***}6{\cdot}0 \pm 0{\cdot}17^{e}$‖	$^{NS}2{\cdot}6 \pm 0{\cdot}09^{cd}$‖
6	UTSI	12	5	$15 \pm 1{\cdot}7^{def}$	$13 \pm 0{\cdot}92^{cd}$	$7{\cdot}6 \pm 0{\cdot}24^{a}$¶	$3{\cdot}0 \pm 0{\cdot}13^{abc}$
7	$ATSI_3$	12	6	$^{***}27 \pm 1{\cdot}4^{ab}$	$^{NS}15 \pm 2{\cdot}16^{bcd}$	$^{NS}7{\cdot}0 \pm 0{\cdot}20^{abcd}$	$^{NS}2{\cdot}8 \pm 0{\cdot}04^{bcd}$
				Sprague-Dawley rats			
8	UTSI	8	10	$13 \pm 0{\cdot}6^{ef}$	$14 \pm 0{\cdot}83^{cd}$	$6{\cdot}7 \pm 0{\cdot}09^{cd}$	$2{\cdot}7 \pm 0{\cdot}07^{bcd}$
9	$ATSI_3$	8	10	$^{***}23 \pm 2{\cdot}4^{bc}$‡	$^{**}18 \pm 1{\cdot}12^{ab}$‡	$^{***}6{\cdot}1 \pm 0{\cdot}07^{c}$‡	$^{NS}2{\cdot}7 \pm 0{\cdot}10^{bcd}$
10	UTSI	12	5	$18 \pm 2{\cdot}1^{cd}$	$13 \pm 0{\cdot}96^{cd}$	$7{\cdot}4 \pm 0{\cdot}26^{ab}$	$3{\cdot}1 \pm 0{\cdot}08^{ab}$
11	$ATSI_3$	12	5	$^{*}28 \pm 2{\cdot}6^{a}$	$^{NS}16 \pm 0{\cdot}97^{abc}$	$^{NS}6{\cdot}4 \pm 0{\cdot}40^{de}$††	$^{NS}2{\cdot}7 \pm 0{\cdot}21^{bcd}$¶
12	UTSI§	8	5	$9 \pm 1{\cdot}1^{f}$	$11 \pm 1{\cdot}1^{d}$	$6{\cdot}5 \pm 0{\cdot}20^{de}$	$2{\cdot}9 \pm 0{\cdot}17^{bcd}$
13	$ATSI_3$	8	5	$^{***}19 \pm 0{\cdot}8^{cd}$	$^{*}16 \pm 1{\cdot}5^{abc}$	$^{NS}6{\cdot}0 \pm 0{\cdot}08^{e}$	$^{NS}2{\cdot}5 \pm 0{\cdot}11^{d}$
14	UTL	8	7	$12 \pm 0{\cdot}8^{ef}$	$14 \pm 0{\cdot}97^{cd}$	$7{\cdot}1 \pm 0{\cdot}23^{abc}$	$3{\cdot}1 \pm 0{\cdot}13^{ab}$
15	ATL	8	7	$^{NS}16 \pm 1{\cdot}6^{de}$	$^{NS}13 \pm 0{\cdot}90^{cd}$	$^{NS}6{\cdot}8 \pm 0{\cdot}12^{cd}$	$^{NS}3{\cdot}0 \pm 0{\cdot}10^{abc}$
17	UTL + Alphacel (1:1)	8	6	$^{***}21 \pm 1{\cdot}5^{cd}$	$^{NS}12 \pm 0{\cdot}86^{cd}$	$^{NS}7{\cdot}0 \pm 0{\cdot}25^{bcd}$	$^{NS}3{\cdot}3 \pm 0{\cdot}20^{a}$

GPT = Glutamic–pyruvic transaminase BUN = Blood urea nitrogen UTSI = Untreated soya protein isolate
$ATSI_3$ = Alkali-treated soya protein isolate (containing 1·27% lysinoalanine)
UTL = Untreated lactalbumin ATL = Alkali-treated lactalbumin

Values are means ± SEM. Letter superscripts indicate groups not significantly different by Duncan's Multiple Range Test. Means without a superscript in common differ at the 5% confidence level. Nonhomogeneous 'outlier' values are excluded from the table and indicated in the footnotes below. Significant differences of each group from its corresponding control group (fed the identical untreated diet for the same time) by the Student's *t*-test are indicated by the superscript to the left of the mean value: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; NS, not significantly different ($P > 0{\cdot}05$).

†See Table 1.

‡Data exclude one rat for which the GPT value was 115 mU/ml and the BUN value was 44 mg%. Total protein value of group 9 was based on eight samples.

§Pair-fed with group 13.

‖n = 9 (insufficient sample from one rat in group).

¶n = 4.

††n = 3.

treated protein, with the exception of a single Sprague-Dawley rat fed $ATSI_3$ for 8 wk, which had markedly elevated GOT, GPT, and ornithine carbamoyl transferase (OCT) values. With the exception of this single animal, OCT values did not differ significantly among test groups. No significant differences were noted in the liver *N*-demethylase activity between groups fed alkali-treated rather than untreated protein.

Red and white blood cells counts made on Sprague-Dawley and Wistar rats fed $ATSI_3$ or untreated soya isolate after 2, 11, 23, 44 and 56 days on the diet, and on pair-fed Sprague-Dawley rats fed the same diet for 56 days, did not reveal any differences among dietary groups. A lower value for the white blood cell count in Sprague-Dawley rats fed 20% $ATSI_2$ ($3{\cdot}6 \pm 0{\cdot}4 \times 10^3/mm^3$, n = 5) than in those fed untreated soya isolate ($6{\cdot}2 \pm 0{\cdot}4 \times 10^3/mm^3$, n = 5) had been observed in a previous experiment, but a similar difference was not observed in this, more extensive, experiment.

Urinary and faecal lysinoalanine excretion

A 10-day balance study was performed to determine the fate of ingested lysinoalanine in rats (Table 3). Animals fed alkali-treated soya protein were found to excrete more faecal nitrogen and slightly less fat than pair-fed controls. The nitrogen data are in agreement with the reduced PER and digestibility values determined in different groups of animals. Approximately 33% of the ingested lysinoalanine was excreted in the faeces. All faecal lysinoalanine was bound, since acid hydrolysis was required to release faecal lysinoalanine. The greatest part of the remaining 67% of the ingested lysinoalanine could not be accounted for.

Liver and blood analysis did not reveal any measurable concentration of lysinoalanine. In the kidneys of animals fed alkali-treated soya protein for 8 wk, however, a small storage of lysinoalanine was found, which accounted for approximately 0·6% of the lysinoalanine ingested in one day or 1·7% of the amount of lysinoalanine disappearing from the gut per day.

When some animals on the $ATSI_3$ diet were changed to a lysinoalanine-free diet and the urinary lysinoalanine was measured, it was found that lysinoalanine excretion continued up to 37–75 hr after the change (Fig. 4). The rate of excretion was very small after the tenth hour. The total quantity excreted

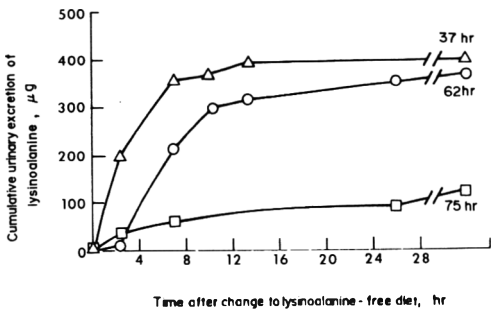

Fig. 4. Urinary excretion of lysinoalanine in Sprague-Dawley rats fed alkali-treated soya protein diet and then changed to a lysinoalanine-free diet. Each curve represents a different animal.

accounted for 0·7–2·7% of the quantity disappearing from the gut daily.

When the urinary lysinoalanine excretion was measured over a 12-hr period in rats fed diets containing alkali-treated lactalbumin, $ATSI_3$, or mixture of untreated and alkali-treated lactalbumin providing varying amounts of lysinoalanine, it was found that the amount of lysinoalanine excreted increased with dietary lysinoalanine levels (Table 3). Rats fed alkali-treated lactalbumin had the highest dietary lysinoalanine content and excreted the highest amount of lysinoalanine. This is noteworthy since it suggests that lysinoalanine is at least as well-absorbed from alkali-treated lactalbumin, which induced very little karyomegaly, as from alkali-treated soya protein. The quantity of lysinoalanine excreted accounted for 1–7% of that ingested per day. There was a wide variability in the amount of lysinoalanine excreted among the animals tested.

Most of the urinary lysinoalanine was free. A small increase in the urinary lysinoalanine was observed after acid-hydrolysis of the urine (Table 3) indicating that a portion of the urinary lysinoalanine was in a bound form. Evidence has been presented (Finot, Bujard & Arnaud, 1976) that some of the lysinoalanine is acetylated in the rat kidney before excretion.

Urinary and faecal excretion and storage in the kidney only accounted for approximately 35% of the ingested lysinoalanine. The remainder could be metabolized either in the animal's body or by the intestinal flora. Experiments with ^{14}C labelled lysinoalanine (Finot *et al.* 1976) indicated that the intestinal flora extensively metabolized lysinoalanine to CO_2 which was expired; and Leegwater (1978) has recently reported a previously unidentified major urinary metabolite.

DISCUSSION

The above results help to clarify a number of issues regarding the relationship between dietary lysinoalanine content and the development of nephrocytomegaly.

We, like Woodard and co-workers (Woodard, 1969; Woodard & Short, 1973; Reyniers, Woodard & Alvarez, 1974; Woodard *et al.* 1975) and Newberne and co-workers (Newberne, Rogers & Wogan, 1968; Newberne & Young, 1966), have consistently observed nephrocytomegaly in rats fed alkali-treated soya protein (containing 0·69% to 1·3% lysinoalanine) at a level of 20% in the diet. Until recently, de Groot and co-workers at the Central Institute for Nutrition and Food Research (CIVO), Zeist, The Netherlands, had consistently reported finding no nephrocytomegaly when similarly-treated soya protein of equal lysinoalanine content was fed to rats (de Groot & Slump, 1969; de Groot *et al.* 1976; de Groot, Slump, van Beek & Feron, 1976). A number of possible explanations for this discrepancy have been discussed in the literature (Feron *et al.* 1977; Gould & MacGregor, 1977; O'Donovan, 1976; Struthers, Dahlgren & Hopkins, 1977). In an attempt to resolve this discrepancy, we supplied Feron *et al.* with a sample of alkali-treated soya protein that we had found to produce nephrocytomegaly in our laboratory ($ATSI_3$, Table 1).

This sample contained no detectable free lysinoalanine (less than 10 ppm) and a very low level (approximately 60 ppm) of trichloroacetic-acid-extractable (low-molecular-weight) material (L. van Beek, personal communication, 1977). Their results with this protein ($ATSI_3$) that we supplied have been published (Feron *et al.* 1977). They fed this protein to CIVO Wistar-derived rats, incorporated into the diet routinely used at the CIVO and in a diet essentially identical with that which we used in the present work.

Nephrocytomegaly was observed in rats fed the latter diet, but only slight nephrocytomegaly in one of five rats fed this protein at the same level in the CIVO diet. Since both diets contained 2630 ppm lysinoalanine, supplied by the identical protein, it is clear that the composition of the diet was a major determinant of the development of nephrocytomegaly.

Feron *et al.* (1977) believe the dietary difference responsible for preventing the development of nephrocytomegaly to be the addition of 10% untreated casein to the CIVO diet. This idea is supported by our present findings with this same alkali-treated soya protein (Table 1, Fig. 2). We observed marked nephrocytomegaly upon feeding this protein for 8 wk to Sprague–Dawley and Wistar rats (Fig. 1, 2). Feeding a dietary level of 12% of this protein, supplemented with an additional 8% untreated lactalbumin, provided 1752 ppm lysinoalanine in the diet but did not induce nephrocytomegaly, even though identically treated soya protein providing 1370 ppm did induce nephrocytomegaly. Further, the relatively high lysinoalanine yield from milder alkali treatment of lactalbumin permitted feeding a much better quality protein with a much higher lysinoalanine content than that of the alkali-treated soya protein. Only minimal *pars recta* karyomegaly was found in rats fed 20% alkali-treated lactalbumin providing 4970 ppm dietary lysinoalanine, while 10% alkali-treated lactalbumin with 10% untreated lactalbumin did not produce nephrocytomegaly despite a dietary lysinoalanine content of 2490 ppm. Thus, there is clearly no simple correlation between dietary lysinoalanine content and the degree of nephrocytomegaly observed. We believe all the above findings are consistent with the hypothesis that a diet with an adequately balanced protein source has a marked protective effect against the development of nephrocytomegaly from alkali-treated protein. It is not yet known if these findings extend to free lysinoalanine.

We think it unlikely that the lysinoalanine in the lactalbumin used was less available to the rat than the lysinoalanine in the soya protein because the nitrogen digestibility of alkali-treated lactalbumin and soya protein were comparable (Table 2), and the urinary lysinoalanine excretion was considerably higher in rats fed alkali-treated lactalbumin than in those fed alkali-treated soya protein (Table 3). The development of nephrocytomegaly, then, is dependent on a number of factors beyond simple available dietary lysinoalanine content. A number of these factors have previously been discussed in detail (Gould & MacGregor, 1977).

The strain difference between the CIVO Wistar-derived rats used by de Groot and co-workers and the Sprague-Dawley-derived rats used by Woodard and co-workers, by Newberne and co-workers, and by ourselves is apparently not an important factor in the divergent findings reported by these groups in the past. As mentioned above, Feron *et al.* (1977) found nephrocytomegaly in CIVO Wistar-derived animals when $ATSI_3$, supplied by us was incorporated into a diet essentially identical with that which we used; they did not, however, observe nephrocytomegaly when this same protein was incorporated into the CIVO standard diet used in the earlier negative studies at CIVO. The reason for the failure of the CIVO group to observe nephrocytomegaly in the past must therefore be attributed to the diet used and not to an insensitivity of CIVO rats. Further, we found an identical shift in the size distribution of *pars recta* nuclei in the affected outer medullary stripe* of Wistar and Sprague-Dawley rats when the same $ATSI_3$-containing diet was fed to both strains (Fig. 2). Feron *et al.* (1977) also compared the sensitivity to free lysinoalanine of Sprague-Dawley and two lines of Wistar-derived rats used at the CIVO and found no major differences.

Struthers *et al.* (1977) have reported finding a marked species difference between Sprague-Dawley and Wistar rats fed alkali-treated soya protein. There were certain differences in the method of preparation of the alkali-treated protein, and in the composition of the experimental diet, employed by these workers and that used by ourselves and others cited above. The description of the lesions observed by these workers is somewhat at variance both with the morphological appearance of the lesions we observed in these experiments and with those described by Woodard (1969) and Woodard & Alvarez (1967). Nonetheless, their results indicate that at least certain lines of a particular strain may vary in their sensitivity to alkali-treated protein-induced renal alterations. This, however, is almost certainly not the reason why the CIVO group failed to observe nephrocytomegaly in their earlier experiments.

Our negative findings in mice are consistent with the known insensitivity of mice to lysinoalanine-induced nephrocytomegaly (de Groot *et al.* 1976). It is noteworthy, however, that high dietary levels of free lysinoalanine do induce nephrocytomegaly in mice (Feron *et al.* 1977), and that the relatively sensitive method of scoring of karyomegaly which we used did reveal an incidence of nuclei with a diameter greater than 8·4 μm which was more than twice as high in the mice receiving alkali-treated soya protein as that in the mice receiving the untreated protein. The probability that a variation of this size could have occurred by chance was very close to the 5% conventionally accepted as statistically significant. It may be that a very slight degree of karyomegaly was in fact produced in the mice, and that it could be observed as statistically significant if larger groups of animals were used, or if more cells were scored per animal. It should be noted that the method by which we score the degree of karyomegaly is very much more sensi-

*Note that the most widely accepted term for the zone of the kidney in which the *pars recta* predominates is the outer stripe of the outer medulla, or the outer medullary stripe (Tisher, 1976; Trump & Bulger, 1968; Woodard *et al.* 1975). This zone has been referred to by some authors as the inner stripe of the cortex.

tive than the subjective comparisons used by others in the past. Further, the feeding time we used in the experiments with mice was arbitrarily set at the time of the maximum expression of karyomegaly in the rat, and is not necessarily the optimal time to observe this response in the mouse. It would be informative to examine larger groups of mice and record the results as a function of the time during which the alkali-treated protein diet was fed using the method of scoring karyomegaly used in these experiments.

The reduced feed efficiency and poor growth performance of the animals fed alkali-treated soya protein is consistent with previously reported findings (de Groot & Slump, 1969), but the altered blood-chemistry values we observed have not previously been noted. The significance of the elevated GPT and BUN values and the decreased plasma protein in the animals fed alkali-treated soya protein is not entirely clear. Elevated GPT can be indicative of liver or kidney damage, but is not specific (Mattenheimer, 1971). Restricting food to one half the *ad lib.* level has been reported to result in increased GPT in rats (Schwartz, Tornaben & Boxill, 1973). We observed an elevation of GPT similar to that observed in the rats fed alkali-treated soya protein when half of the dietary protein was replaced with Alphacel (Table 5, Group 17), but not in animals pair-fed untreated soya protein at a dietary intake equal to that of the *ad lib.* quantity ingested by the rats fed alkali-treated soya protein (Table 5, Group 12). Neither of these two groups had elevated BUN or decreased plasma protein values. Nevertheless, it is possible that these changes are due to the altered nutritional balance of the diets used rather than to organ-specific effects of lysinoalanine or other compounds formed in the alkali-treated protein.

The nature of the decreased nutritional quality of the alkali-treated soya protein, and the relationship of the development of nephrocytomegaly to the available amino-acid balance in the diet, are not well understood. The particular amino acid or combination of amino acids responsible for the prevention of the kidney abnormalities, and how it relates to the mechanism of induction of the lesion attributed to lysinoalanine, remains to be established. Newberne & Young (1966), and Newberne *et al.* (1968) showed that supplementation with methionine, choline and vitamin B_{12} could prevent similar kidney lesions produced by Alpha Protein, a protein we now know to have contained lysinoalanine and to have had a nutritionally inadequate amino acid composition. Woodard & Alvarez (1967) could not confirm these findings with alkali-treated soya protein, nor could van Beek (personal communication, 1977) demonstrate a reduction in the severity of the nephrocytomegaly induced by free lysinoalanine by supplementing with these nutrients.

Chemical analyses of our alkali-treated soya protein and alkali-treated lactalbumin (Table 4) showed that the alkali-treated lactalbumin had higher lysine, leucine, methionine, threonine, valine, isoleucine and tyrosine than did the alkali-treated soya protein. In addition to these differences, the animals fed alkali-treated lactalbumin had a higher food intake than did the animals fed the alkali-treated soya protein, thereby accentuating the higher nutrient intake. Hair loss, which has been reported to result from lysine deficiency (Gershoff *et al.* 1958), was observed in the animals fed alkali-treated soya protein, but not in those fed alkali-treated lactalbumin. Chemical analysis, however, is not always a good indicator of nutritional damage. For example, de Groot & Slump (1969) found the nutritional damage from hot alkali treatment of soya protein to be greater than could be accounted for by either losses in essential amino acids or decreased overall nitrogen digestibility. Nonetheless, the above amino acids which differ markedly in the alkali-treated soya protein and alkali-treated lactalbumin should be systematically examined to determine whether the balance of these amino acids modulates the development of nephrocytomegaly, and therefore explains the marked difference in the severity of the lesions obtained with these two proteins. We have already found that supplementation of the diet used in this study with a combination of 0·8% L-lysine, 0·55% L-arginine and 0·45% L-threonine did not significantly diminish the severity of the karyomegaly after 8 wk on the 20% alkali-treated soya protein diet (unpublished results).

It should also be mentioned explicitly that we do not consider it proved that the lysinoalanine formed in the alkali-treated protein is the sole, or necessarily even the principal, factor responsible for the induction of nephrocytomegaly. The present evidence does, however, favour the hypothesis that lysinoalanine is the factor in alkali-treated protein that induces nephrocytomegaly, but that nutritional factors strongly modulate the development of the lesions. Recent findings, however, demonstrate that lysinoalanine is not unique among the potential products of alkaline treatment in its ability to induce *pars recta* lesions with a similarity to the lesions induced by alkali-treated proteins. Ornithinoalanine (Feron *et al.* 1977), fructoselysine (Erbersdobler, von Wangenheim & Hänichen, 1978) and D-serine (D. H. Gould & J. T. MacGregor, unpublished data) all produce lesions of the *pars recta* with certain features in common with the lysinoalanine-induced lesion. As discussed previously (Gould & MacGregor, 1977), other products of alkali treatment could also play a role in the development of nephrocytomegaly. The problem is obviously complex, and it may be some time before a full appreciation of all the factors governing the development of nephrocytomegaly in animals ingesting alkali-treated proteins is realized.

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FORTHCOMING PAPERS

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

Reduction of sulphonated water-soluble azo dyes by micro-organisms from human faeces. By T. Watabe, N. Ozawa, F. Kobayashi and H. Kurata.

The effect of sodium saccharin in the diet on caecal microflora. By R. L. Anderson and J. J. Kirkland.

Subchronic studies in rats fed octenyl succinate modified food starch. By M. L. Buttolph and P. M. Newberne.

Effect of free fatty acids on aflatoxin production in a synthetic medium. By E. Priyadarshini and P. G. Tulpule.

An investigation of the genetic toxicology of irradiated foodstuffs using short-term test systems. 1. Digestion *in vitro* and the testing of digests in the *Salmonella typhimurium* reverse mutation test. By B. J. Phillips, E. Kranz, P. S. Elias and R. Münzner.

Liver-enzyme induction in lindane- and captan-treated rats. By Y. B. Mikol, F. Roux, F. Decloître and E. P. Fournier.

Absorption and metabolism of three phthalate diesters by the rat small intestine. By R. D. White, D. E. Carter, D. Earnest and J. Mueller.

Accumulation of polychlorinated dibenzofurans in the livers of monkeys and rats. By H. Kuroki, Y. Masuda, S. Yoshihara and H. Yoshimura.

Aniline: Evidence for an enterogastric cycle in the rat. By R. D. Irons, E. A. Gross and E. L. White.

Absorption, excretion, metabolism and cardiovascular effects of beetroot extract in the rat. By C. Krantz, M. Monier and B. Wahlström.

The percutaneous absorption of lead-203 in humans from cosmetic preparations containing lead acetate, as assessed by whole-body counting and other techniques. By M. R. Moore, P. A. Meredith, W. S. Watson, D. J. Sumner, M. K. Taylor and A. Goldberg.

Hydroxycitronellal—a survey of consumer patch-test sensitization. By R. J. Steltenkamp, K. A. Booman, J. Dorsky, T. O. King, A. S. Rothenstein, E. A. Schwoeppe, R. I. Sedlak, T. H. F. Smith and G. R. Thompson.

Citral—a survey of consumer patch-test sensitization. By R. J. Steltenkamp, K. A. Booman, J. Dorsky, T. O. King, A. S. Rothenstein, E. A. Schwoeppe, R. I. Sedlak, T. H. F. Smith and G. R. Thompson.

Cinnamic alcohol—a survey of consumer patch-test sensitization. By R. J. Steltenkamp, K. A. Booman, J. Dorsky, T. O. King, A. S. Rothenstein, E. A. Schwoeppe, R. I. Sedlak, T. H. F. Smith and G. R. Thompson.

Oestrogenic activity of soya-bean products. By H. M. Drane, D. S. P. Patterson, B. A. Roberts and N. Saba. (Short paper).

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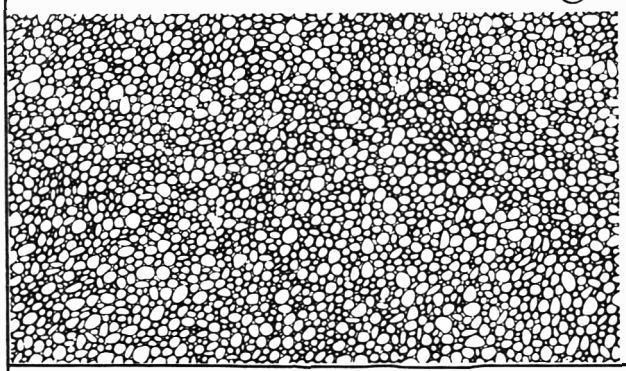

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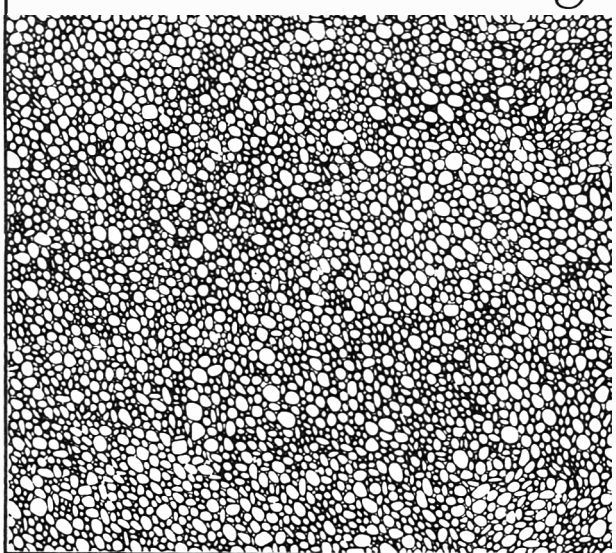

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