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INFORMATION SECTION

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Research Section

ACTION OF DYES AND INDICATORS ON RAT-LIVER REGENERATION

L. L. GERSHBEIN

Northwest Institute for Medical Research, 5656 West Addison Street, Chicago, IL 60634, USA

(*Received 7 May 1981*)

Abstract—When various dyes and indicators were fed to partially hepatectomized male rats as supplements in a basal diet for 10 days after surgery, liver regeneration was stimulated by 1-phenylazo-2-naphthol and by 1-(*p*-phenylazophenylazo)-2-naphthol, as such or with methyl substituents on the phenyl rings, and also by cresyl violet, D & C Yellow No. 11 and methyl orange. The efficacy of the azonaphthols stemmed from the activity of the metabolic intermediate, 1-amino-2-naphthol, which was tested in rats at a level of 0·20% (as the hydrochloride). The presence of a nitro group or two methoxy groups on the phenyl ring of 1-phenylazo-2-naphthol or of sulphonic acid groups in either of the azonaphthol series led to compounds that were ineffective. The stimulants generally elicited liver enlargement when fed to intact rats for the same interval. In contrast to *p*-dimethylaminoazobenzene, which was ineffective at the level (0·20%) tested, the sulphonic acid derivative sodium *p*-dimethylaminoazobenzenesulphonate (methyl orange) was, as stated above, stimulatory. Differences in the molecular configuration of azo dyes are thus reflected in their action on regenerating liver. Sulphanilic acid, a prominent metabolite of several azo dyes, proved inactive in partially hepatectomized males at a level of 0·20%.

INTRODUCTION

Although the effects of some colouring agents on organs such as the liver have been reported, few studies have dealt with the action of dyes on regenerating liver. Stowell (1949) compared cellular changes in the livers of intact, partially hepatectomized and 4-dimethylaminoazobenzene-fed rats over a period of 5–6 months, and Mironescu, Encut, Mironescu & Liciu (1968) described nucleolar alterations in the regenerating liver of rats injected ip 4-aminoazobenzene or one of the carcinogens, 4-dimethylaminoazobenzene and 3′-methyl-4-dimethylaminoazobenzene. The last named dye, in conjunction with X-irradiation, caused changes in the proportion of various liver-cell populations in operated rats (Webber & Stich, 1965). In contrast to the stimulatory activity of several other carcinogenic hydrocarbons, 4-dimethylaminoazobenzene fed at 0·10% for 10 days did not affect the extent of liver regeneration (Gershbein, 1958). The maximal uptake of indocyanine green by the liver has been shown to be a good gauge of the functional mass of the intact or regenerating organ (Rikkers, Brown & Moody, 1973). In another study, neutral red uptake by the hepatocytes of regenerating rat liver reached a peak 24 hr after injection (Isanin, Kuznetsova & Yakovlev, 1974).

This paper presents liver findings in partially hepatectomized rats fed diets supplemented with dyes representing several prominent types. Special attention was directed to the 1-azo-2-naphthol derivatives, in view of their widespread usage and the tumorigenic properties of several aminonaphthols. Where a stimulatory response was evident, the effect was generally ascertained on the wet and dry liver-weight percentages in intact rats over the same observation period.

EXPERIMENTAL

All diets were prepared by trituration of the test agent with Purina Rat Chow meal (Ralston Purina Co., Inc., St. Louis, MO) on a weight basis. The sources of the dyes are presented in Tables 1 and 2. The dyes were of industrial grade; a number of the indicators were of high purity. With a few exceptions, the dye contents were in the 90–99% range. Oil Red O was also administered by gavage, a mull of dye and 20% of its weight of gum tragacanth being suspended in water to give a 5% suspension of the dye; the controls were force-fed the corresponding aqueous gum mixture.

Charles River (COBS® outbred and Sprague–Dawley descended) and Holtzman male rats were partially hepatectomized under ether by the method of Higgins & Anderson (1931), two-thirds of the organ being removed. The liver portions were dried to constant weight at 100 C. The animals were placed in individual cages and administered water and diet *ad lib.* for an interval of 10 days, or for shorter periods in a few series. They were then killed with ether and the entire livers were extirpated and dried to constant weight. Very small sections of tissue were reserved for microscopic examination. The increment, or the amount of liver regenerating, was calculated from the dry weights by subtracting the product of the weight

Table 1. *Liver- and body-weight data for partially hepatectomized male rats fed diet containing a dye or indicator*

Diet†‡	Dietary concn (%, w/w)	No. of rats/group	Body weight (g)§ Initial	Body weight (g)§ Terminal	Liver increment (g)§	t§
Series 29-A						
Control	—	15	268 ± 11·7	313 ± 11·3	2·496 ± 0·141	
Oil Red O	0·20	15	290 ± 11·2	293 ± 9·9	3·261 ± 0·120	4·14**
	0·075	15	262 ± 7·3	294 ± 5·7	2·877 ± 0·119	2·06*
Series 29-B						
Control	—	18	314 ± 9·3	330 ± 8·7	2·032 ± 0·076	
Cresyl violet	0·10	15	308 ± 12·1	301 ± 9·6	2·531 ± 0·089	4·12**
Methyl orange	0·20	14	319 ± 8·4	328 ± 6·1	2·509 ± 0·090	4·08**
Series 29-D						
Control	—	14	235 ± 6·3	297 ± 8·7	1·894 ± 0·087	
D & C Red No. 17	0·050	13	239 ± 7·7	288 ± 9·6	2·214 ± 0·119	2·19*
Series 29-E						
Control	—	14	231 ± 7·7	307 ± 7·8	2·073 ± 0·075	
D & C Red No. 17	0·30	9	238 ± 8·0	298 ± 7·3	2·997 ± 0·165	5·74**
Series 29-G						
Control	—	10	261 ± 7·6	297 ± 5·4	2·369 ± 0·071	
Sudan IV	0·10	13	267 ± 7·4	302 ± 8·4	3·112 ± 0·175	3·54**
D & C Yellow No. 11	0·060	12	258 ± 8·0	292 ± 8·3	2·639 ± 0·132	1·82
Series 29-H						
Control	—	15	271 ± 5·6	286 ± 7·0	2·071 ± 0·091	
D & C Yellow No. 11	0·15	12	268 ± 7·4	277 ± 5·4	2·814 ± 0·134	5·73**
Series 29-K						
Control	—	11	257 ± 5·3	277 ± 6·1	2·242 ± 0·107	
Janus Green B	0·050	12	255 ± 5·1	264 ± 5·0	1·857 ± 0·075	2·98**
Series 29-L						
Control	—	16	299 ± 7·2	310 ± 5·3	2·238 ± 0·074	
Sulphanilic acid	0·20	11	282 ± 8·9	309 ± 9·8	2·264 ± 0·085	0·22
Series 29-M						
Control	—	14	247 ± 5·0	263 ± 4·2	1·855 ± 0·042	
Morton Orange Y	0·10	8	246 ± 6·4	236 ± 7·1	2·561 ± 0·154	5·43**
Calco Oil Red A1700	0·20	13	235 ± 5·2	230 ± 3·5	2·696 ± 0·139	5·95**
Series 29-P						
Control	—	13	256 ± 5·2	268 ± 9·1	1·871 ± 0·095	
Morton Orange Y	0·050	12	254 ± 4·1	261 ± 3·1	2·205 ± 0·095	2·46*
Series 29-Q						
Control	—	14	292 ± 9·9	314 ± 9·6	1·906 ± 0·079	
Calco Oil Scarlet BL	0·20	10	297 ± 7·9	267 ± 6·4	2·419 ± 0·122	3·72**
Oil Red 113	0·20	13	299 ± 9·6	312 ± 8·4	2·632 ± 0·113	4·78**
Oil Orange 204	0·20	11	288 ± 7·3	281 ± 7·6	2·989 ± 0·165	6·89**
Series 29-R						
Control	—	14	242 ± 3·3	283 ± 4·6	2·319 ± 0·072	
Citrus Red No. 2	0·20	14	248 ± 7·3	282 ± 7·1	2·532 ± 0·109	1·64
Toluidine red	0·20	15	250 ± 5·2	278 ± 5·8	2·328 ± 0·103	0·02
Series 29-S						
Control	—	16	293 ± 3·1	306 ± 4·6	2·001 ± 0·068	
1-Amino-2-naphthol.HCl	0·20	13	298 ± 2·6	296 ± 4·1	2·363 ± 0·118	2·19*
Series 29-T						
Control	—	10	299 ± 5·3	297 ± 5·2	1·746 ± 0·079	
Oil Red O	0·20	12	289 ± 7·6	273 ± 6·5	1·918 ± 0·093	1·38
Cresyl violet	0·10	11	300 ± 7·0	275 ± 7·1	1·856 ± 0·080	0·97
Series 29-U‖						
Control	—	8	250 ± 6·6	248 ± 9·5	1·489 ± 0·100	
Oil Red O	800 mg/kg‖	9	243 ± 8·8	253 ± 6·9	1·892 ± 0·091	2·86**

†Control diet (ground Purina Rat Chow) alone or with the name supplement was fed *ad lib.* for a 10-day period after surgery to male Charles River rats, except in series 29-M (Holtzman rats), series 29-T (Holtzman rats for 5 days) and series 29-U (see below).

‡Sources of test materials (with Colour Index—3rd ed., 1971—numbers and synonyms in brackets) were as follows: Oil Red O (26125, Solvent Red 27), cresyl violet and Sudan IV (26105, Scarlet Red, Solvent Red 24) from Allied Chemical Corp., New York; D & C Yellow No. 11 (47000, CI Solvent Yellow 33, Quinoline SS), D & C Red No. 17 (26100, CI Solvent Red 23, Sudan III) and Citrus Red No. 2 (12156) from Buffalo Color Corp., West Paterson, NJ; Calco Oil Red A1700 (26120, Solvent Red 26) and Calco Oil Scarlet BL (12140, Solvent Orange 7, Sudan II) from American Cyanamid Co., Bound Brook, NJ; Oil Red 113 (12150, Solvent Red 1) and Oil Orange 204 (12100, Solvent Orange 2) from Passaic Color & Chemical Co., Paterson, NJ; Morton Orange Y (12055, Solvent Yellow 14, Sudan I) from Morton Chemical Co., Chicago, IL; toluidine red (12120, Pigment Red 3) and 1-amino-2-naphthol hydrochloride from Aldrich Chemical Co., Milwaukee, WI; Janus Green B (11050) from Sigma Chemical Co., St. Louis, MO. Methyl orange from Merck & Co., Inc., Rahway, NJ, and sulphanilic acid from Mallinckrodt Inc., St. Louis, MO, were of reagent grade.

§Values are means ± SEM for the numbers of animals shown. Significant *t* values are marked with asterisks: $*P < 0.05$; $**P < 0.01$.

‖Series 29-U involved gavage administration of a gum tragacanth mu.l, providing the rats in the test group with four 50-mg/kg doses of Oil Red O over 5 days. Control and test rats were fed ground basal chow and killed on day 6.

Table 2. *Dietary supplements without significant effect on liver regeneration in partially hepatectomized male rats*

Colouring	CI (1971) no.	Source*	Dietary concn tested† (%)
Acriflavin	46000	S	0·030
Alizarin Red S	58005	A	0·20
Amaranth	16185	S	0·30
Aniline blue	42775	S	0·20
Aurintricarboxylic acid	—	EK	0·30
Brilliant cresyl blue	51010	A	0·10
Brilliant sulphaflavine	56205	A	0·10
Congo red	22120	A	0·20
Crystal violet	42555	AC	0·030
D & C Blue No. 6 (CI Vat Blue 1)	73000	BC	0·30
D & C Green No. 5 (CI Acid Green 25; Alizarin Cyanin Green F	61570	BC	0·30
D & C Orange No. 4 (CI Acid Orange 7; Orange 11)	15510	BC	0·30
D & C Red No. 19 (CI Basic Violet 10; Rhodamine B)	45170	BC	0·20
D & C Red No. 33 (CI Acid Red 33; Acid Fuchsin D)	17200	BC	0·30
D & C Red No. 37 (CI Solvent Red 49; Rhodamine B stearate)	45170	BC	0·20
D & C Violet No. 2 (CI Solvent Violet 13; Alizarin Purple SS)	60725	BC	0·30
Ext. D & C Yellow No. 7 (CI Acid Yellow 1; Naphthol Yellow S)	10316	BC	0·30
D & C Yellow No. 7 (CI Acid Yellow 73 (free acid); fluorescein)	45350	BC	0·30
D & C Yellow No. 8 (CI Acid Yellow 73 (Na salt); uranine)	45350	BC	0·30
D & C Yellow No. 10 (CI Acid Yellow 3; Quinoline Yellow WS	47005	BC	0·30
Diazo Red RC	37120	S	0·20
Evans blue	23860	AC	0·20
FD & C Blue No. 1 (CI Food Blue No. 2: Brilliant Blue FCF)	42090	BC	0·20
FD & C Blue No. 2 (CI Food Blue No. 1; sodium indigo disulphonate	73015	BC	0·20
FD & C Green No. 3 (CI Food Green No. 3; Fast Green FCF)	42053	BC	0·20
FD & Red No. 40 (CI Food Red No. 17; Allura® Red)	16035	BC	0·40
FD & C Yellow No. 5 (CI Food Yellow No. 4; tartrazine	19140	BC	0·20
FD & C Yellow No. 6 (CI Yellow No. 4; Sunset Yellow FCF)	15985	BC	0·20
Janus Green B	11050	S	0·025‡
Malachite green base	42000	S	0·067
Methyl green	42585	S	0·030
Methylene blue chloride	52015	M	0·10
Methyl red	13020	AC	0·20
p-Dimethylaminoazobenzene, methyl yellow	—	EK¶	0·20
Naphthol blue black	20470	H	0·20
Neutral red	50040	S	0·20
Nigrosin	50420	S	0·30
Phenol red, phenolsulphonphthalein	—	A	0·20
Phloxine B	45410	S	0·20
Ponceau S	15635	M	0·10
Safranin O	50240	AC	0·060
Tetrazolium blue	—	S	0·15
Tetrazolium red	—	S	0.050
Thiazole Yellow G	19540	H	0·20
Toluidine Blue O	52040	S	0·20
Xylene Brilliant Cyanin G	42655	KK	0·075

*Sources of the colourings were: Aldrich Chemical Co. (A); Allied Chemical Corp. (AC); Buffalo Color Corp. (BC); Eastman Kodak Co., Rochester, NY (EK); Harleco, Gibbstown, NJ (H); K & K Laboratories, Inc., Plainview, NY (KK); Matheson, Coleman & Bell, Norwood, OH (M); Sigma Chemical Co. (S).

†Diets were fed for 10 days to groups of 14–17 partially hepatectomized male Charles River rats.

‡At a dietary level of 0·05%, this dye depressed liver regeneration (see Table 1).

¶Subsequently recrystallized.

at surgery and the factor 0·46 from the weight at autopsy (Gershbein & Labow, 1953). Rats losing more than about 15% of their initial body weight or displaying gross fatty infiltration of the liver were excluded.

Diets containing agents that stimulated liver regeneration were administered also to intact rats for 10 days, after which time the livers were removed and the ratios of wet and dry liver to total body weights were determined and compared with those of the controls. A few diets were also tested in intact and operated animals over a period of 5 days, the liver-weight percentages and hepatic increments, respectively, being ascertained.

RESULTS

Body weights and liver increments for rats fed diets supplemented with Oil Red O (0·075 and 0·20%), cresyl violet (0·10%), methyl orange (0·20%), D & C Red No. 17 (0·050 and 0·30%), Sudan IV (0·10%), Calco Oil Red A1700 (0·20%), Morton Orange Y (0·050 and 0·10%), Calco Oil Scarlet BL (0·20%), Oil Red 113 (0·20%), Oil Orange 204 (0·20%) and 1-amino-2-naphthol hydrochloride (0·20%), together with the Fisher *t*-values for the comparison of the liver data against the respective controls, appear in Table 1. The increments were significantly elevated in these dye-fed groups, as in the rats fed D & C Yellow No. 11 at 0·15% but not at 0·060% (series 29-G and 29-H). Weight losses were extensive in rats administered the aminonaphthol hydrochloride at 0·35 or 0·50%. Sulphanilic acid, a metabolite of several azo dyes, Citrus Red No. 2 and toluidine red, each fed at 0·20%, proved ineffective. Oil Red O force-fed in doses of 50 mg/rat on days 1, 3, 4 and 5 after surgery, with the rats being killed on day 6, likewise stimulated the regenerating liver. In contrast to the 10-day treatments, the liver incremental increases for groups fed Oil Red O (0·20%) or cresyl violet (0·10%) for 5 days were not significant (series 29-T).

Table 1 also presents data for Janus Green B, which depressed liver regeneration at 0·050%. When the level was lowered to 0·025% (Table 2), the mean increment was within the control range. Depressed regeneration was also observed with such dyes as crystal violet (at 0·060%), methylene blue chloride (0·20%) and Safranin O (0·20%) together with excess weight loss, but diets containing 0·030, 0·10 and 0·060% of these three, in the order stated, were better tolerated and the mean values simulated those of the controls (Table 2). The latter table lists dyes of various types that did not modify the basal response when fed to partially hepatectomized rats at the designated levels.

In the series of studies on intact rats, the ratios of both the wet and dry liver weight to total body weight in immature rats fed Morton Orange Y (0·050%), Calco Oil Scarlet BL (0·10%) and Oil Orange 204 (0·10%) and in heavier animals administered Oil Red O (0·20%), cresyl violet (0·10%), Sudan IV (0·20%), methyl orange (0·20%) and D & C Yellow No. 11 (0·15%) for 10 days were markedly higher than in the controls (Table 3). As in the operated series, Citrus Red No. 2 (0·20%), toluidine red (0·20%) and crystal violet (0·030%) were ineffective. In contrast to the data in Table 1, Oil Red 113 at a somewhat lower level (0·15%) was inactive. The rations were better tolerated by the intact rats: organ enlargement ensued when Oil Red O (0·20%) or Sudan IV (0·10%) was fed for 5 days.

Microscopic examination of liver sections (stained

Table 3. *Data for intact male rats fed diets supplemented with dyes*

Diet†	Dietary concn (% w/w)	No. of rats	Body weight (g)		Liver weight (g/100 g body weight)			
			Initial	Terminal	Wet weight	*t*	Dry weight	*t*
Series 29-V								
Control		15	113 ± 2·5	180 ± 3·9	4·996 ± 0·106		1·422 ± 0·031	
Morton Orange Y	0·050	14	115 ± 2·4	176 ± 4·3	5·740 ± 0·142	4·25**	1·601 ± 0·039	3·65**
Calco Oil Scarlet BL	0·10	14	115 ± 2·4	154 ± 3·5	6·656 ± 0·159	8·78**	1·866 ± 0·041	8·71**
Oil Orange 204	0·10	14	115 ± 2·8	162 ± 5·3	6·330 ± 0·105	8·89**	1·769 ± 0·028	8·26**
Oil Red 113	0·15	14	114 ± 1·9	167 ± 4·2	5·224 ± 0·110	1·49	1·491 ± 0·029	1·60
Toluidine red	0·20	14	114 ± 1·8	182 ± 3·3	5·238 ± 0·104	1·62	1·509 ± 0·034	1·89
Citrus Red No. 2	0·20	14	113 ± 2·0	173 ± 4·9	5·008 ± 0·089	0·10	1·415 ± 0·030	0·16
Series 29-W								
Control		16	361 ± 8·5	382 ± 9·0	3·541 ± 0·044		1·080 ± 0·016	
Crystal violet	0·030	15	364 ± 9·1	389 ± 5·9	3·703 ± 0·073	1·95	1·069 ± 0·032	0·31
Oil Red O	0·20	16	368 ± 7·8	389 ± 7·8	4·358 ± 0·123	6·24**	1·240 ± 0·043	5·70**
Cresyl violet	0·10	15	373 ± 12·3	379 ± 9·9	4·363 ± 0·099	7·75**	1·282 ± 0·031	5·94**
Methyl orange	0·20	16	371 ± 8·1	389 ± 6·7	3·948 ± 0·055	5·81**	1·155 ± 0·020	3·00**
Series 29-X								
Control		14	277 ± 3·7	324 ± 5·2	4·189 ± 0·097		1·186 ± 0·024	
D & C Yellow No. 11	0·15	14	280 ± 3·7	323 ± 4·6	4·842 ± 0·092	5·88**	1·401 ± 0·039	4·78**
Sudan IV	0·20	14	278 ± 3·3	313 ± 5·0	5·308 ± 0·098	8·11**	1·537 ± 0·028	9·49**
Series 29-Y								
Control		15	254 ± 3·4	276 ± 3·9	4·215 ± 0·084		1·236 ± 0·024	
Sudan IV	0·10	15	256 ± 3·6	276 ± 4·6	5·083 ± 0·106	6·53**	1·491 ± 0·034	6·07**
Oil Red O	0·20	15	254 ± 3·6	268 ± 3·8	5·035 ± 0·093	6·56**	1·462 ± 0·026	6·46**

†Control diet alone or with the named supplement was fed *ad lib.* to intact male Charles River rats, except in series 29-V (Holtzman rats) and series 29-Y (5-day feeding period). Sources of the colourings are given in a footnote to Table 1.
Values are means ± SEM for the numbers of rats shown. Significant *t* values are marked with asterisks: $^{**}P < 0{\cdot}01$.

with haematoxylin–eosin) revealed no outstanding pathology in comparison with the corresponding controls. The fresh livers showed the presence of pigment, especially with several of the ingested 'active' dyes.

DISCUSSION

Of the dyes and indicators investigated in the current study, relatively few stimulated regeneration of the liver. Four of the active agents were 1-phenylazo-2-naphthols (Morton Orange Y, Oil Orange 204, Oil Red 113 and Calco Oil Scarlet BL) and four were 1-(*p*-phenylazophenylazo)-2-naphthols, the unsubstituted one being D & C Red No. 17 (Sudan III) while the compounds with two, three and four methyl groups on the benzene rings were Sudan IV, Calco Oil Red A1700 and Oil Red O, respectively (Table 4). The aminonaphthoaminophenazonium acetate, cresyl violet, can also be viewed as related to 1-naphthylamine, whereas D & C Yellow No. 11 is a condensation product of quinaldine and phthalic anhydride, and methyl orange is a simple azo dye. sodium *p*-dimethylaminoazobenzenesulphonate.

In the metabolism of azo compounds, reductive fission occurs at the azo grouping with the production of an amine. The cleavage is not greatly influenced by the presence of sulphonic acid groups on the nucleus. In addition to the reduction, hydroxylation of the aromatic nucleus occurs, as noted from the excretory products. Presumably, the hydroxylation is prevented by the presence of sulphonic acid groups at either side of the amino group or where more than two methyl groups occur (Daniel, 1962). Enzymes for these reactions emanate from the liver and, especially, the intestinal flora. The literature of azo compound metabolism in the mammal has been reviewed by Walker (1970).

Following reductive cleavage the azonaphthalene hepatic stimulants considered here would yield the 1-naphthylamine derivatives, and the carcinogenic properties of such agents as well as of 2-naphthylamines have been investigated by several workers. Radomski (1961) noted the urinary excretion of 1-amino-2-naphthyl sulphate following single oral doses of Citrus Red No. 2 and D & C Red No. 14 (1-xylylazo-2-naphthol), and another derivative, Ponceau S, was shown to produce liver tumours on chronic feeding (Grice, Mannell & Allmark, 1961). With regard to the naphthylamines and liver, the writer (1975) reported a stimulation of regeneration in operated rats by 2-naphthylamine at 0·35%, borderline stimulation with 1-naphthylamine at 0·25% and a definite response with *N*-phenyl-1-naphthylamine (0·50%); both the 1- and 2-naphthylamines caused liver enlargement in intact rats at a dietary level of 0·35%. Of great pertinence is the current demonstration that liver regeneration is stimulated by 1-amino-2-naphthol fed as the hydrochloride at 0·20%. The latter was relatively toxic when added to the feed at higher levels.

The effect of azonaphthols on rat-liver regeneration is summarized in Table 4. With 1-phenylazo-2-naphthols, the absence of substituents on the phenyl ring (Morton Orange Y) or the occurrence of 1 or 2 methyl groups or a single methoxy group did not alter the hepatotrophic activity. However, the presence of a nitro group (as in toluidine red) or the introduction of a second methoxy group (Citrus Red No. 2) led to 'inert' compounds. The presence of sulphonic acid groups gave rise to a level of activity within the control range, as exemplified by D & C Orange No. 4, FD & C Red No. 40, FD & C Yellow No. 6 and amaranth and, among the 1-(*p*-phenylazophenylazo)-2-naphthols, by Ponceau S, in contrast to D & C Red No. 17 and its three methyl derivatives. The negative findings with the sulphonates may be due to the general observation that such compounds are poorly absorbed from the gastro-intestinal tract, but this does not apply to the lipid-soluble dyes (Childs, Nakajima & Clayson, 1967; Gingell, Bridges & Williams, 1969).

The feeding of agents that were active in operated rats caused increases in both wet and dry liver weights in intact rats, and again Citrus Red No. 2 and toluidine red were ineffective. When tested in immature rats (series 29-V; Table 3), the mono-methoxy dye, Oil Red 113, also elicited increases in wet and dry liver-weight percentages that were not statistically significant.

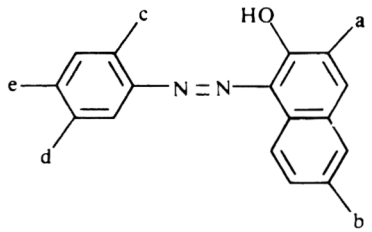
It must be stressed that the diets used in this study contained fairly low levels of the various dyes to allow for better toleration and to guard against excessive weight losses, since inanition will depress the liver increment. In addition, the observation period was quite short. With longer intervals of dye administration, several liver changes have been reported. Liver enlargement and liver cell adenomas were observed on feeding Ponceau S to rats at levels of up to 5·0% for 10 months (Ikeda, Horiuchi, Furuya & Omori, 1966), and liver weight increased and hepatic fatty changes were prominent when Ponceau MX was fed at 2·0% for 90 days (Hall, Lee & Fairweather, 1966). Although no organ enlargement nor any evidence of carcinogenicity could be discerned by others on feeding the latter dye at 1·0% for 2 yr (Grasso, Lansdown, Kiss *et al.* 1969), the earlier liver lesions were interpreted as neoplastic by Bonser & Roe (1970). Liver mass was increased on administration of diets containing up to 2·0% toluidine red for 90 days to intact rats (Graham & Davis, 1968) and on feeding another type of dye, Rhodamine B or D & C Red No. 19 (Webb, Hansen, Desmond & Fitzhugh, 1961), although both proved ineffective in the current experiments on operated rats. Other types of toxicity inherent in Rhodamine B and azo dyes have been elucidated recently. Thus D & C Orange No. 17, D & C Red No. 19 and D & C Red No. 36 have been found to be mutagenic (Brown, Dietrich & Bakner, 1979; Muzzall & Cook, 1979), although the activity of D & C Red No. 19 appeared to be due to an impurity.

The triphenylmethane dyes screened (aniline blue, aurintricarboxylic acid, crystal violet, malachite green, methyl green, Xylene Brilliant Cyanin G, FD & C Blue No. 1 and FD & C Green No. 3) did not modify the response of operated control rats. Previously, some of the dyes had been shown to produce sarcomas on sc injection into rats (Gross, 1961) and to inhibit enzymes involved in protein synthesis (Liao, Horwitz, Huang & Grollman, 1975). Of several water-soluble agents fed over a period of 2 yr, Guinea Green B caused liver enlargement and was identified as a hepatocarcinogen (Hansen, Long, Davis *et al.* 1966).

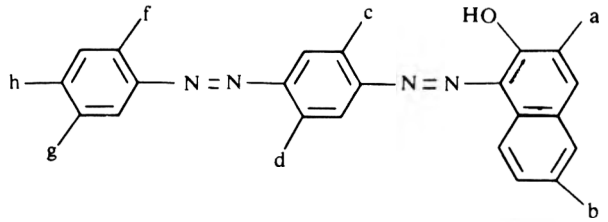
Table 4. *Summary of liver findings in partially hepatectomized rats fed azonaphthol derivatives*

Compound (and CI [1971] no.)	Identity of group located* at: a	b	c	d	e	f	g	h	Effect†
Amaranth (16185)‡									−
1-Amino-2-naphthol hydrochloride									+
1-Phenylazo-2-naphthol colourings									
Morton Orange Y (12055)	—H	—H	—H	—H	—H				+
Oil Orange 204 (12100)	—H	—H	$—CH_3$	—H	—H				+
Oil Red 113 (12150)	—H	—H	$—OCH_3$	—H	—H				+
Citrus Red No. 2 (12156)	—H	—H	$—OCH_3$	$—OCH_3$	—H				−
Calco Oil Scarlet BL (12140)	—H	—H	$—CH_3$	—H	$—CH_3$				+
Toluidine red (12120)	—H	—H	$—NO_2$	—H	$—CH_3$				−
D & C Orange No. 4 (15510)	—H	—H	—H	—H	$—SO_3Na$				−
FD & C Red No. 40 (16035)	—H	$—SO_3Na$	$—OCH_3$	$—CH_3$	$—SO_3Na$				−
FD & C Yellow No. 6 (15985)	—H	$—SO_3Na$	—H	—H	$—SO_3Na$				−
1-(*p*-Phenylazophenylazo)-2-naphthol colourings									
D & C Red No. 17 (26100)	—H	—H	—H	—H		—H	—H	—H	+
Sudan IV (26105)	—H	—H	$—CH_3$	—H		$—CH_3$	—H	—H	+
Calco Oil Red A1700 (26120)	—H	—H	$—CH_3$	$—CH_3$		$—CH_3$	—H	—H	+
Oil Red O (26125)	—H	—H	$—CH_3$	$—CH_3$		$—CH_3$	$—CH_3$	—H	+
Ponceau S (15635)	$—SO_3Na$	$—SO_3Na$	$—SO_3Na$	—H		—H	—H	$—SO_3Na$	−

*1-Phenylazo-2-naphthol type:



1-(*p*-Phenylazophenylazo)-2-naphthol type:



†Derived from series of studies described above: + = significant increase in the liver increment; − = response similar to that in control rats.
‡Trisodium salt of 1-(4-sulpho-1-napthylazo)-2-naphthol-3,6-disulphonic acid.

The lack of a stimulatory effect on liver regeneration in the tests on triphenylmethane dyes now reported was to be expected, considering the refractory character of the parent hydrocarbon, triphenylmethane, when fed at levels of up to 1·0% (Gershbein, 1975).

Butter yellow (*p*-dimethylaminoazobenzene) has been the subject of a number of tumour studies. Du Plooy & Dijkstra (1971/1972) noted that during its metabolism, azo-group reduction, *C*-hydroxylation and *N*-demethylation occurred prior to the steps involved in the carcinogenic action, namely, *N*-hydroxylation and binding to macromolecular components of the cell. The regenerating liver has been used in studies of the binding of such azo compounds and discrete components (Sugimoto & Terayama, 1972; Warwick, 1967) and the possible role of *N*-hydroxylation, esterification and reaction with nucleic acids has been amplified by Lin, Miller & Miller (1975). As with the dye fed at 0·10%, which had little effect on liver regeneration (Gershbein, 1958), a level of 0·20% was non-stimulatory and was not too well tolerated.

In contrast to *p*-dimethylaminoazobenzene, the introduction of a sulphonic acid group para to the azo group yields methyl orange, which stimulated liver regeneration at 0·20% and also increased wet and dry liver weight in intact males. These findings appear to be contrary to those observed with the active azonaphthols as such and with their sulphonic acid-substituted derivatives, which proved ineffective. Although a comprehensive study has not been undertaken at this stage of the effect of such groups as the sulphonic acid substituents, the parent compounds differed in activity, with *p*-dimethylaminoazobenzene being ineffective or tending to depress liver regeneration. Accordingly, such behaviour must reflect the mode and extent of metabolism and binding with cell components, aspects which vary with the molecular configuration among azo dyes. Sulphanilic acid, which is a metabolite of methyl orange and is also excreted into the urine of rats fed tartrazine and related dyes (Ryan, Welling & Wright, 1969), did not affect the control increment when given at a dietary level of 0·20%.

As discussed above, many of the dyes studied here are poorly absorbed and present little liver involvement. Recently, Wahlström, Blennow & Krantz (1979) investigated the action of quinoline yellow on the perfused rat liver and noted that excretion into the bile occurred within 3 hr and little metabolism by the liver could be discerned, findings similar to those following iv administration of the colouring into the anaesthetized animal.

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DISPARATE *IN VIVO* AND *IN VITRO* IMMUNOMODULATORY ACTIVITIES OF RHODAMINE B

J. A. WESS and D. L. ARCHER

Division of Microbiology, Food and Drug Administration, Department of Health and Human Services, Cincinnati, OH 45226, USA

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Abstract—At nontoxic concentrations (1–10 μg/ml) rhodamine B (*N*-[9-(2-carboxyphenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride; D & C Red No. 19; CAS no. 81-88-9), a low-molecular-weight dye, suppressed the primary *in vitro* plaque-forming-cell response of BDF_1 mouse spleen cells both to the thymus-dependent antigen, sheep red blood cells and to the thymus-independent antigen, *E. coli* 0127:B8 lipopolysaccharide. Suppression was effective when the D & C Red No. 19 was added at any time up to 48 hr of culture. Rhodamine B at concentrations from 1 to 10 μg/ml significantly suppressed mitogen-induced proliferation of both B- and T-lymphocytes *in vitro*. BDF_1 mice exposed to 50 and 100 ppm dye in their drinking water for 14 days prior to splenectomy gave a depressed *in vitro* plaque-forming-cell response to sheep red blood cells. In contrast, a significant enhancement of antibody response resulted when mice were exposed to the dye and sheep-red-blood-cell antigen was administered *in vivo*. Mitogen-induced B- and T-lymphocyte proliferation was also enhanced in mice exposed to the dye for 14 days.

INTRODUCTION

The ability of various food additives to suppress the primary *in vitro* thymus-dependent and thymus-independent responses has been reported (Archer, Smith & Bukovic-Wess, 1978). The phenolic antioxidant food additive BHA appeared to affect both B- and T-lymphocytes (Archer, Bukovic-Wess & Smith, 1977a) while gallic acid appeared to affect only macrophage-dependent T-lymphocyte function(s) (Archer, Bukovic-Wess & Smith, 1977b; Archer *et al.* 1978). Although 2ME reversed GA-inhibited primary *in vitro* anti-SRBC response (Archer *et al.* 1977b), it did not reverse the similar response of BHA-inhibited cultures (Archer & Wess, 1979).

In our initial *in vitro* screening of various chemicals, D & C Red No. 19 (Rhodamine B; *N*-[9-(2-carboxyphenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]-*N*-ethylethanaminium chloride; CAS no. 81-88-9; mol wt 479), a dye commonly used in drugs, cosmetics, and toiletries, exerted immunosuppression. This study was designed to determine whether the immunological activity of D & C Red No. 19 was the same under *in vitro* and *in vivo* conditions.

EXPERIMENTAL

Animals and chemicals. Six- to 8-wk-old BDF_1 (C57Bl/6 × DBA/2) female mice were obtained from Laboratory Supply Co., Indianapolis, IN. Animals were given autoclavable mouse chow 5010C (Ralston Purina, St. Louis, MO) and acidified water (pH 3) *ad lib.* D & C Red No. 19 was obtained from the Certification Branch, Division of Color Technology, FDA. Appropriate dilutions of the dye were prepared in Eagle's Minimum Essential Medium (Gibco, Grand Island, NY) and added in 10–100 μl amounts to the culture plates. Similar quantities of culture medium without the dye were added to control plates. The 2ME, obtained from Matheson, Coleman and Bell, Norwood, OH, was 99% pure. $N^6,O^{2'}$-Dibutyryl c-AMP was purchased from Calbiochem, San Diego, CA.

Primary IgM response. Dissociated mouse spleen cells were prepared according to the method described by Mishell & Dutton (1967) at a concentration of $1{\cdot}5 \times 10^7$ cells in 1 ml of RPMI 1640 (Microbiological Associates, Bethesda, MD) with 10% foetal calf serum. Erythrocytes from a single sheep were supplied by Colorado Serum Co., Denver, CO, and used at a concentration of 3×10^6 SRBC/culture to immunize cultures *in vitro* for the thymus-dependent response. *Escherichia coli* 0127:B8, described by Johnson, Peeler & Hall (1968), was used as the thymus-independent antigen. On day 5 of culture, direct plaque-forming cell assays were performed according to the method of Cunningham & Szenberg (1968) for SRBC. The microscope slide modification of Golub, Mishell, Weigle & Dutton (1968) was used to determine the anti-0127:B8 LPS response. The cells in each culture were counted in a haemacytometer, and culture viability was determined by trypan blue dye exclusion. Data are presented as means ± SEM of triplicate cultures. The background PFC level never exceeded 6% of the SRBC-induced PFC response.

Feeding study. Four groups of mice (six mice/group) were exposed for a 2-wk period to various concen-

Abbreviations: c-AMP = Adenosine 3'5'-cyclic monophosphoric acid; BHA = butylated hydroxyanisole; Con A = concanavalin A; FDA = Food and Drug Administration; LPS = lipopolysaccharide; 2ME = 2-mercaptoethanol; PFC = plaque-forming cell(s); SEA = staphylococcal enterotoxin A; SRBC = sheep red blood cell(s).

Table 1. *D & C Red No. 19 and the immune response* in vitro

Amount of D & C Red No. 19 in culture (μg)*	Percentage inhibition of direct PFC response in culture	
	Anti-SRBC	Anti-0127:B8
0·1	11	57
1·0	38	65
10·0	80	93

PFC = plaque-forming cell SRBC = sheep red blood cell
*Dye was added at time of antigen addition.

trations (0, 10, 50 and 100 ppm) of D & C Red No. 19 in their drinking water. The mice were then killed by cervical dislocation and their spleens were removed. The effect of *in vivo* exposure to dye on the anti-SRBC PFC response was examined by setting up 5-day cultures (according to Mishell & Dutton, 1967) and determining direct PFC/culture. In another set of experiments, 4 days before completion of the 2-wk exposure period, the mice were injected ip with 0·2 ml of 2% SRBC suspension in normal saline (Koller, Exon & Roan, 1976). Four days later the mice were killed by cervical dislocation, their spleens were removed, and a direct PFC assay for SRBC was set up according to the method of Cunningham & Szenberg (1968). Cell recovery was determined using a Coulter Counter (Model Z_B).

Mitogen assays. Con A, crystallized three times was purchased from Miles Laboratories, Elkhart, IN. SEA was produced by the Microbial Biochemistry Branch, Division of Microbiology, FDA, Cincinnati, OH; the purity of SEA is documented elsewhere (Schantz, Roessler, Woodburn *et al.* 1972). Lipopolysaccharide of *E. coli* 0127:B8 was obtained from Difco Laboratories, Detroit, MI. To induce DNA synthesis, mitogens were added to lymphocyte cultures at the following concentrations, as determined by preliminary titrations: 0·5 μg SEA/0·2 ml, 1 μg Con A/0·2 ml, or 1 μg LPS/0·2 ml. Tritiated thymidine, purchased from New England Nuclear, Boston, MA, was added at a final concentration of 0·5 μCi per well. The method used for determination of DNA synthesis is reported elsewhere (Archer, Smith, Ulrich & Johnson, 1979). Mitogen assays were performed by direct addition of D & C Red No. 19 to normal splenic lymphocytes *in vitro*, or by use of spleens from mice exposed *in vivo* for 14 days to various concentrations of the dye.

RESULTS AND DISCUSSION

Effects of D & C Red No. 19 on immune responses in vitro

Direct addition of D & C Red No. 19 to cultures (Mishell & Dutton, 1967) of BDF_1 spleen cells at the time of antigen addition resulted in 38–80% suppression of the anti-SRBC PFC response over a concentration range of 1·0–10 μg/ml (Table 1). The thymus-independent (anti-0127:B8 LPS) response of BDF_1 splenocytes appeared even more susceptible than the SRBC PFC response to suppression induced by the dye. As little as 0·1 μg/ml of D & C Red No. 19 resulted in 57% suppression of the anti-0127:B8 LPS PFC response (Table 1): 65 and 93% suppression were obtained with 1·0 and 10 μg/ml, respectively.

D & C Red No. 19 exerted its full suppressive effect on the *in vitro* anti-SRBC PFC response through 48 hr of culture and even delaying the addition of the dye for 72 hr of culture resulted in about 60% suppression (Table 2). It appears unlikely that early events such as antigen presentation or lymphocyte induction are affected by D & C Red No. 19, since macrophage-dependent T-lymphocyte factors required for induction must be present before 48 hr of culture (Dutton, 1976). The results indicate that 2ME, which replaces some macrophage functions *in vitro* (Chen & Hirsch, 1972; Lemke & Opitz, 1976), failed to reverse anti-SRBC PFC suppression induced by the dye (Table 3). This finding suggests that D & C Red No. 19 does not interfere with lymphocyte surface sulphydryl groups, as sulphydryl reagents suppress the thymus-dependent PFC response, but the suppression is reversible by 2ME and other thiols (Johnson, 1980). The addition of 75–100 μg/culture of c-AMP also failed to reverse suppression of the anti-SRBC PFC response induced by the dye (data not shown). This finding suggests that D & C Red No. 19 does not act *via* suppressor T-cells because c-AMP has been shown to effect reversal of suppressor T-cell effects (Johnson, Blalock & Baron, 1977).

D & C Red No. 19 at concentrations from 1·0 to 10 μg/ml inhibited polyclonal T- and B-cell mitogen-induced proliferation in a dose-dependent manner

Table 2. *Kinetics of the D & C Red No. 19 suppression of the immune response* in vitro

Time after addition of dye (hr)*	Direct primary anti-SRBC response (PFC/culture)	
	Experiment A	Experiment B
0 (control)†	11150 ± 1250	3333 ± 595
0	400 ± 200	33 ± 33
24	400 ± 300	0
48	500 ± 200	0
72	4300 ± 500	567 ± 187

PFC = plaque-forming cell
SRBC = sheep red blood cell
*Dye (10 μg) was added at the time of antigen addition.
†Cultures to which no dye was added.
Values are means of triplicate cultures ± SEM.

Table 3. *Effect of addition of 2-mercaptoethanol on D & C Red No. 19-inhibited anti-SRBC and anti-0127:B8 PFC response*

Culture content (with/without 2ME)*	Direct response (PFC/culture)	
	Control	D & C Red No. 19 (10 μg)*
Anti-SRBC response		
Experiment 1		
2ME not added	15900 ± 1300	1650 ± 750
2ME added	16600 ± 500	2450 ± 550
Experiment 2		
2ME not added	11350 ± 850	700 ± 200
2ME added	8400 ± 100	250 ± 50
Anti-0127:B8 response		
2ME not added	580 ± 50	15 ± 15
2ME added	270 ± 50	20 ± 20

PFC = Plaque-forming cell 2ME = 2-Mercaptoethanol
SRBC = Sheep red blood cell
*2ME (5×10^{-5} M) and dye were added at the time of antigen addition.
Values are means of triplicate cultures ± SEM.

(Table 4). At a level of 5 μg (0·2 ml culture containing 2×10^5 cells) the dye inhibited both T-cell proliferation induced by Con A and SEA (98 and 92%, respectively) and B-cell proliferation induced by LPS (98%). Inhibition by D & C Red No. 19 of both the thymus-dependent and thymus-independent PFC responses may be due to the ability of the dye to suppress lymphocyte proliferation. The mechanism by which D & C Red No. 19 exerts its anti-proliferative effect is unknown. Although Rhodamine 6G (which is structurally related to Rhodamine B) is a potent inhibitor of oxidative phosphorylation (Gear, 1974), D & C Red No. 19 has no known effect on energy-related function(s). It is possible, however, that D & C Red No. 19 is altered *in vitro* to a form which inhibits energy production.

Effects of D & C Red No. 19 on immune responses in vivo

When D & C Red No. 19 was added to the drinking water of BDF_1 mice at concentrations of 0, 10, 50 and 100 ppm for 14 days the water consumption of all the treated groups was identical to that of the controls. In all experiments, the mice were killed on day 5, and the spleens were prepared for culture. Figure 1 shows the effect of *in vivo* D & C Red No. 19 treatment on the *in vitro* anti-SRBC PFC response. Analysis of variance and Duncan's (1955) multiple range test showed that the response of mice given 50 and 100 ppm of the dye was significantly suppressed ($P < 0.05$) compared with that of control animals. No suppression of the anti-SRBC PFC response was observed at the 10 ppm level. In contrast, Fig. 2 shows that mice exposed *in vivo* to D & C Red No. 19 and immunized with SRBC 4 days before splenectomy (day 10 of dye exposure) demonstrated a significantly enhanced ($P < 0.05$) PFC response (with means of 1264, 1359, 1858 PFC/10^6 viable cells for 10, 50 and 100 ppm, groups, respectively) compared with untreated controls (mean 509). Exposure to antigen *in vivo* would be the normal sensitization (or immunization) route; thus, an enhanced antibody response is more likely to occur in humans. *In vitro* exposure of lymphoid cells to D & C Red No. 19 resulted in suppression (Table 1) and *in vivo* exposure to both D & C Red No. 19 and antigen resulted in enhancement of the immune response. This finding suggests that D & C Red No. 19 is metabolized *in vivo* to a form that

Table 4. *The effect of* in vitro *D & C Red No. 19 treatment on mitogen-induced DNA synthesis in mouse spleen cells*

D & C Red No. 19 treatment (μg)*	DNA synthesis ([3H]thymidine incorporated, cpm) in the presence of...			
	Control	Staphylococcal enterotoxin A	Concanavalin A	Lipopolysaccharide
0	2044 ± 49	8612 ± 710	19753 ± 340	16418 ± 158
0·1	1899 ± 383	10316 ± 528	15650 ± 1176	14614 ± 1311
1·0	973 ± 152	5672 ± 1159	6312 ± 754	7538 ± 605
5·0	183 ± 28	653 ± 63	338 ± 15	354 ± 53
10·0	134 ± 41	291 ± 74	159 ± 25	158 ± 45

*Amount of dye (μg/0·2 ml of culture containing 2×10^5 cells).
Values are means of triplicate cultures ± SEM.

Table 5. *The effect of* in vivo *D & C Red No. 19 treatment of BDF$_1$ mouse spleen-cell proliferation and mitogen-induced DNA synthesis*

D & C Red No. 19 treatment* (ppm)	DNA synthesis ([^{3}H]thymidine incorporated, cpm)				
	Without added mitogen		In the presence of...		
	Experiment A	Experiment B†	Staphylococcal enterotoxin A	Concanavalin A	Lipopolysaccharide
0	590 ± 267	2055 ± 267	5637 ± 968	5280 ± 1019	7963 ± 1239
10	1541 ± 450	9318 ± 168	14254 ± 1550	40049 ± 5441	19660 ± 4618
50	1986 ± 640	9095 ± 321	16534 ± 1994	37759 ± 7218	20269 ± 2138
100	1109 ± 258	—	—	—	—

*Mice were exposed to the various concentrations of dye in their drinking water for 14 days.
†Control values for mitogen studies.
Values are means of 18 replicate cultures ± SEM.

enhances the immune response, while the non-metabolized D & C Red No. 19 (*in vitro*) suppresses lymphocyte function. Although the reason for the suppression observed in the *in vitro* PFC assay of animals exposed to D & C Red No. 19 *in vivo* is unclear, it could reflect an alteration of the metabolite of the dye due to cell culture conditions which render it suppressive; however, other explanations are equally plausible.

The effect of *in vivo* exposure of mice to D & C Red No. 19 on spleen-cell proliferation was also studied. The normal rate of DNA synthesis in splenic lymphocytes increased significantly after exposure to the dye *in vivo* (Table 5). The lack of a significant difference between the DNA synthesis rates observed in the 10 and 50 ppm does groups suggests that the stimulatory threshold may have been reached; no dose lower than 10 ppm was included in these studies.

D & C Red No. 19 enhanced the DNA synthesis rate of resting lymphocytes, and led to enhanced mitogen-induced DNA synthesis (Table 5). The rates of T- and B-cell mitogen-induced DNA synthesis were significantly higher in spleen cells from mice treated with the dye. The DNA synthesis rates for SEA- and LPS-induced dye exposed cells appeared to be additive; that is, the sums of the DNA synthesis rates induced by the dye alone plus SEA or LPS alone were roughly equal to the rates induced by combined *in vivo* dye pretreatment and *in vitro* mitogen-induced activation. The DNA synthesis rate resulting from Con A activation of lymphocytes exposed to the dye, however, exceeded the sum of individual treatments.

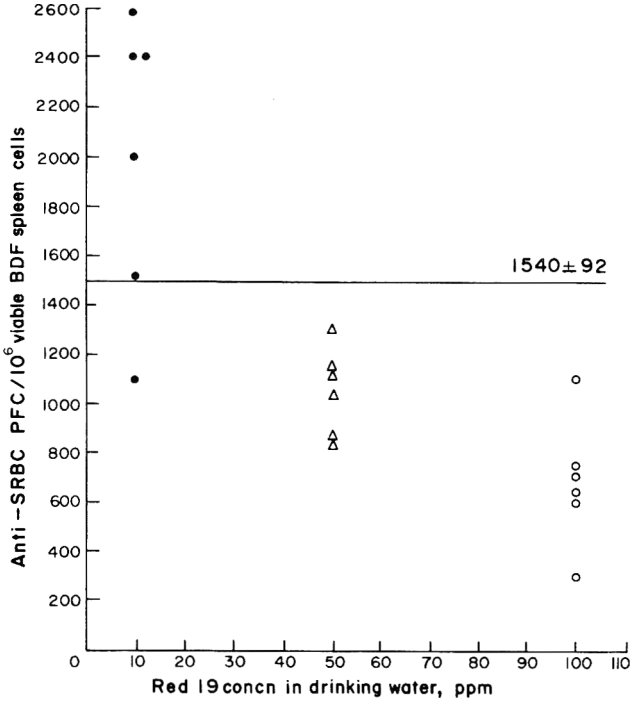

Fig. 1. BDF$_1$ mice (six/group) were exposed to 0, 10, 50 and 100 ppm D & C Red No. 19 in their drinking water for 14 days. The points represent the anti-SRBC PFC response/10^6 viable spleen cells of dye-exposed groups; the response of the non-exposed group is represented by a straight line (mean ± SEM from 12 replicates).

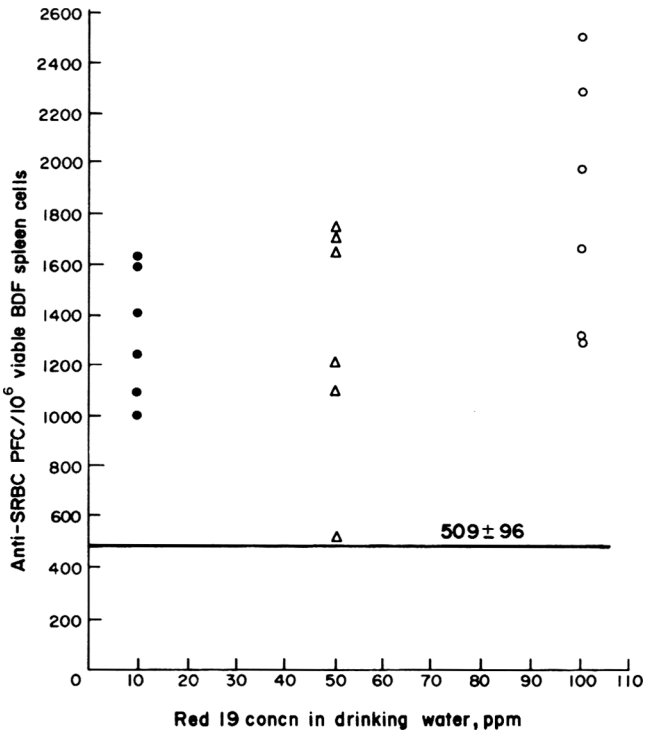

Fig. 2. BDF_1 mice (six/group), were exposed to 0, 10, 50 and 100 ppm D & C Red No. 19 in their drinking water for 14 days, and injected ip on day 10 with 0·2 ml of 2% SRBC suspension in normal saline. The points represent the anti-SRBC PFC response/10^6 viable spleen cells of dye-exposed groups; the response of the non-exposed group is represented by a straight line (mean ± SEM from 12 replicates).

Con A has been shown to activate more T-cell subpopulations than SEA (Archer *et al.* 1979); therefore, D & C Red No. 19 (or a metabolite produced *in vivo*) may act as a co-mitogen for a T-cell subpopulation not activated by SEA. The fact that administration of D & C Red No. 19 *in vivo* potentiates B- and T-cell mitogen-induced proliferation would seem to contradict the data which show depressed *in vitro* antibody synthesis from dye-treated mice (Fig. 1). The longer *in vitro* incubation time used in the Mishell-Dutton system (5 days *v.* 2 days in mitogen studies) may provide a partial explanation of the apparent discrepancy.

The immunopotentiating capabilities of D & C Red No. 19 (or its metabolite) are interesting pharmacologically because an increased or amplified immune response may be desirable in certain clinical situations. The IgE (allergic or reaginic) antibody response is highly thymus-dependent; increased IgE production could exacerbate allergic responses, and this would be undesirable.

This study demonstrates the need for *in vivo* exposure studies to determine the potential immunotoxicity of ingested compounds. The data clearly show that the *in vitro* immunosuppressive activity of a compound does not predict the *in vivo* immunologic activity accurately.

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LONG-TERM TOXICITY STUDY OF QUILLAIA EXTRACT IN RATS*

J. J.-P. DRAKE, K. R. BUTTERWORTH, I. F. GAUNT, J. HOOSON, J. G. EVANS and S. D. GANGOLLI

The British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey SM5 4DS, England

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Abstract—Groups of 48 male and 48 female rats were fed quillaia extract in the diet at levels of 0 (control), 0·3, 1·0 and 3·0% for 2 yr. The material had no adverse effects on death rate, water consumption, serum chemistry or haematological parameters or on the incidence of histopathological findings, including tumours. In the males given the 3% dietary level, the death rate, total leucocyte count at wk 108, incidence of kidney lesions and weights of the kidneys, heart and thyroid were all below the control values. These differences were explicable, however, in terms of a lowered body weight consequent on a decreased food intake. It is concluded that, in rats, quillaia extract fed at levels up to 3·0% in the diet did not have any carcinogenic effect. The no-untoward-effect level established in this study was 3·0% in the diet, approximately equivalent to an intake of 1·5 g/kg/day.

INTRODUCTION

Quillaia extract, an aqueous extract of the dried inner part of the bark of *Quillaia saponaria* Molina and other species of Quillaia (Rosaceae), contains three or possibly four saponins (two major, one minor and one trace constituent) together accounting for about 10% of the extract. Glucose, galactose, arabinose, xylose, rhamnose and two further unidentified sugars are also present. The two major saponins are quillaia sapogenin, which has a triterpenoid structure, and quillaic acid (EEC Scientific Committeee for Food, 1978). The amphipathic properties of saponins, a reflection of the water solubility of the carbohydrate moiety and the lipid solubility of the sapogenin, account for their use as foaming agents and emulsifiers.

In the UK, the Emulsifiers and Stabilisers in Food Regulations 1980 (Statutory Instrument 1980, no. 1833) restrict the use of quillaia extracts to soft drinks, at not more than 200 ppm by weight. This measure endorses the recommendation of the Food Additives and Contaminants Committee (1970 & 1972) that quillaia of British Pharmacopoeial standard should be provisionally acceptable for restricted use in food. The Committee stressed the need for further toxicological data, including long-term feeding studies and studies on possible intestinal irritation. The EEC Scientific Committee for Food (1978) established an acceptable daily intake of 5 mg/kg body weight for the spray-dried extract. Quillaia no longer comes within the scope of the EEC directive on food emulsifiers, stabilizers, thickeners and gelling agents (*Off. J. Europ. Commun.* 1980, **23** (L155), 23); instead it is subject to national regulations.

Gaunt, Grasso & Gangolli (1974) reviewed the toxicological status of quillaia and presented the results of a short-term study in rats. Relative liver weight was reduced in males given 2·0 or 4·0% quillaia in that study, the relative stomach weight was increased in both sexes at the same levels and a no-untoward-effect level of 0·6% in the diet was demonstrated. A long-term study in mice showed no carcinogenic response with dietary levels of up to 1·5% quillaia extract and established a no-untoward-effect level of 0·5% (Phillips, Butterworth, Gaunt *et al.* 1979). This paper describes the final part of this safety evaluation programme, a study involving the long-term dietary administration of quillaia extract to rats.

EXPERIMENTAL

Materials. The sample of quillaia extract used in these studies was supplied by Food Industries Ltd, Bromborough Port, Merseyside. It was a spray-dried aqueous extract of quillaia bark, prepared in such a way that 100 parts by weight of bark yielded approximately 15–18 parts of powdered extract. The extract was stated to contain less that 10% moisture and less than 10% ash (at 550 C). The specification conformed to that given in the UK Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486) and to that in the British Pharmacopoeia (1973).

Animals. Weanling rats of a Wistar-derived strain from a specified-pathogen-free breeding colony (A. Tuck and Son, Rayleigh, Essex) were housed in an air-conditioned room maintained at 20 ± 2 C and were given Spratt's Laboratory Animal Diet No. 1 and water *ad lib.*

Experimental design and conduct

Acceptability of test diets. Two short diet-acceptability studies were conducted. In the first, male rats (255–275 g body weight) were housed in pairs in individual plastics cages with access to both control diet and a second diet containing 0·3, 1·0 or 3·0% quillaia

*Further details of the data from this study are tabulated in BIBRA Research Report No. 1/1978, which can be obtained on request.

extract. The amount of each diet consumed was recorded daily for 21 days. In the second experiment, pairs of male rats (358–395 g body weight) were fed on diet containing either 0 (control), 0·3, 1·0 or 3·0% quillaia extract for 7 days, with daily measurement of body weight and food intake.

Long-term feeding study. Groups of 48 male and 48 female rats were housed four in a cage and fed on diets containing 0 (control), 0·3, 1·0 or 3·0% quillaia extract for 2 yr. They were observed regularly and any rats showing signs of ill health were isolated. These were returned to their cage if their condition improved; otherwise they were killed and autopsied. The rats were weighed at approximately 2-monthly intervals up to 2 yr and the consumption of food and water was measured for the 24-hr period prior to body-weight determinations. At wk 15, 25 and 52, blood was collected from the tail veins of ten male and ten female rats from each of the groups fed diets containing 0, 1·0 or 3·0% quillaia extract. After 108 wk, blood was collected from the aorta of all remaining animals during the post-mortem examination. On all samples, measurements were made of haemoglobin concentration and packed cell volume, together with total erythrocyte and leucocyte counts. Slides for differential leucocyte counts were prepared for all samples, but only those from the control and 3·0% dietary groups were examined.

At wk 13, 24 and 78, urine was collected from ten rats from the controls and the highest level of treatment. It was examined for appearance and microscopic constituents, and semi-quantitative tests for the content of protein, glucose, ketones, bile salts and blood were carried out. Concentration and dilution tests were carried out at these times, involving measurement of the specific gravity and volume of urine produced in a 6-hr period without water, over a 4-hr period commencing after 16 hr without water and over a 2-hr period following a water load of 25 ml/kg body weight. The number of cells present was counted in the 2-hr sample.

Serum separated from blood collected at autopsy was examined for contents of urea, glucose, total protein and albumin, and the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactate dehydrogenase were determined.

Animals that died during the study were autopsied unless this was precluded by advanced autolysis or cannibalism. Those found *in extremis* during the study or surviving beyond wk 106 were killed by exsanguination from the aorta under barbiturate anaesthesia and examined for macroscopic abnormalities. Where possible the animals were deprived of food overnight before killing and the brain, heart, liver, spleen, kidney, stomach, small intestine, caecum (with and without its contents), adrenal glands, gonads, pituitary and thyroid were weighed. Samples of these tissues, together with salivary glands, thymus, various lymph nodes, pancreas, aorta, nasal bones, lungs, trachea, oesophagus when present, colon, rectum, skeletal muscle, spinal cord, sciatic nerve, uterus or prostate and seminal vesicles, urinary bladder, mammary tissue, eye and Harderian gland, and other tissues if they appeared abnormal, were preserved in buffered formalin. Paraffin-wax sections of all available tissues except nasal bones were stained with haematoxylin and eosin for histological examination. Sections of the nasal bones were prepared from the animals on the highest dietary level. Samples of femoral bone marrows were stained, but were not examined in view of the absence of other haematological findings.

RESULTS

Acceptability tests

Rats offered the choice between the control and the 0·3% quillaia diet consumed on average 23 g of the control diet and only 1·3 g of the test diet daily. The corresponding average daily intakes were 23·6 g (control) and 2·0 g (test) when the 1·0% quillaia diet was the alternative and 27·4 g (control) and 0·8 g (test) with 3·0% quillaia. When there was no choice of diet, the average intakes over 7 days were 25, 29, 26 and 21 g/day for rats given diet containing 0, 0·3, 1·0 and 3·0% quillaia, respectively. The corresponding weight gains were 26, 29, 30 and 11 g.

Long-term feeding study

Rats given up to 3% quillaia extract in the diet appeared normal. In the male rats fed 1% of the extract, the number of deaths exceeded those among control animals from wk 73 onwards. However, the values were statistically significant only between wk 85 and 91. There was a trend towards a lower number of deaths in male rats fed 3% quillaia extract (Table 1), the difference being statistically significant at wk 97 and 99.

Male rats fed the highest dietary level had lower body weights throughout the experiment than did control animals (Table 2), the differences being statistically significant in the examinations covering the period 10–22 months. Females on the lowest dietary level had significantly higher body weights during the first 6 months of the study.

In the group of male rats fed 3% quillaia extract in the diet, food consumption was consistently lower than that of the controls (Table 3) and water consumption was lower than in the controls from wk 71 (Table 3). During this latter period, the water intake of the males given 3% quillaia extract remained approximately constant whereas that of the controls increased markedly. The calculated daily intakes of quillaia extract are also shown in Table 3.

At wk 13 and 24 the results of the renal concentration and dilution tests showed no statistically significant differences between the control rats and those given 3% quillaia extract. At wk 78, the urine produced in the 4-hr period following water deprivation for 16 hr by male rats fed 3% quillaia extract was of a higher specific gravity than that of the controls (Table 4). This difference was not observed in females nor in male rats at any other time. The cell excretion rates were similar in the treated and control rats.

There were no statistically significant differences between treated and control animals in the results of serum analyses. The only statistically significant findings ($P < 0·05$) in the red-cell parameters were an increase in haemoglobin level at wk 15 in males fed 1% quillaia (17·5 g/100 ml *v.* 16·5 g/100 ml in the controls) and an increased erythrocyte count at wk 108 in females on the same diet (7·60 *v.* 7·18 million/mm^3). There was no trend in other determinations to sup-

Table 1. *Cumulative totals of deaths in rats fed diets containing 0–3% quillaia extract for up to 2 yr*

Duration of test (wk)	Dietary level (%)...	Total no. of deaths							
		Males				Females			
		0	0·3	1·0	3·0	0	0·3	1·0	3·0
79		2	3	4	1	6	4	4	2
83		2	3	6	1	8	6	5	3
87		3	3	13**	2	9	7	6	7
91		6	5	15*	3	9	9	9	9
95		9	7	17	3	10	10	9	9
99		14	7	19	7*	12	10	9	10
103		16	13	20	9	13	13	10	14

The figures are the total numbers of animals dead or killed *in extremis* from groups of 48. Those marked with asterisks differ significantly from the corresponding control value (chi-square test): $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

port either of these isolated findings. Total leucocyte counts were higher in all treated male rats examined at wk 15 and in those given 3% quillaia at wk 25 (Table 5). In contrast, these counts were lower at wk 108 in both sexes given the highest dietary level. There were no significant alterations in the differential counts except at wk 15, when the neutrophils showed an increase and the lymphocytes a decrease at the 3% level.

The weights of heart, kidneys and thyroid were lower than the control values in male rats fed 3%, and thyroid weights were also lower among males fed 1% quillaia extract (Table 6). These differences were statistically significant, but no such differences were observed in the females. When the weights were expressed in relation to body weight (Table 6) the same organs showed low values compared with the controls although the differences did not reach statistical significance. In the females, weights of the small intestine and caecum were higher than in controls at both the highest and 0·3% dietary level. These groups also showed higher stomach weights. When expressed in relation to body weight, the weights of the stomach, intestine and caecum in these two groups of females remained higher than the control values, but the values for the stomach and small intestine were not statistically significant in the group on 0·3% quillaia. The only other significant changes in relative organ weights were a higher liver weight in females given the 3% level and a lower liver weight in males given 1%.

In general the incidence of histological findings was similar in treated and control animals. The only lesions with incidences greater than those of the controls were fibrosis of the heart and dilatation of the glands of the gastric mucosa in the females fed 0·3% extract (Table 7). A variety of benign and malignant tumours was found (Table 8). Some of the tumours occurred only in the treated groups, but the numbers were small, most were not found in the animals given the highest treatment level and no relation to dose was observed. Thus, for example, the incidences of

Table 2. *Mean body weights of rats fed diets containing 0–3% quillaia extract for up to 2 yr*

Duration of test (wk)	Body weight (g)							
	Males given dietary levels (%) of:				Females given dietary levels (%) of:			
	0	0·3	1·0	3·0	0	0·3	1·0	3·0
4	256	258	256	249*	172	179*	173	175
8	356	365	358	344	219	226*	220	219
16	452	458	454	440	259	269*	263	263
25	503	508	504	493	282	296*	289	288
34	550	554	547	528	302	317	310	305
42	598	604	591	568*	329	337	331	326
48	632	636	621	596*	350	362	356	345
54	655	663	649	618*	379	384	378	366
63	684	689	677	641**	404	407	403	395
71	703	700	697	657**	421	426	422	410
80	712	707	697	660**	435	445	445	417
89	683	670	689	637*	433	451	457	420
106	662	683	662	623	431	445	462	417

Results are means for all survivors in each group. Those marked with asterisks differ significantly (Student's *t* test) from their corresponding controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 3. *Mean food and water consumption and calculated intake of emulsifier by rats fed diets containing 0–3% quillaia extract for up to 2 yr*

Dietary level (%)	Mean intakes at wk: 4	25	54	71	89	106
Food consumption (g/rat/day)						
Males						
0	23·0	21·5	21·1	20·2	18·7	16·8
0·3	22·7	20·2	19·1	19·1	18·8	16·3
1·0	22·0	18·3	18·8	18·8	16·8	18·8
3·0	21·1	19·7	18·7	18·4	18·8	14·5
Females						
0	17·5	17·1	16·8	14·6	14·8	14·7
0·3	16·4	17·1	15·8	15·0	14·1	13·3
1·0	16·1	15·6	15·5	13·3	13·7	17·0
3·0	16·6	14·8	16·0	14·8	13·8	13·6
Water consumption (ml/rat/day)						
Males						
0	31·1	28·1	24·9	31·3	37·3	37·2
0·3	32·2	27·9	28·4	29·6	33·0	30·9
1·0	29·6	25·2	29·1	31·0	33·1	34·5
3·0	31·1	29·5	29·4	27·1	29·4	30·9
Females						
0	27·2	27·7	30·8	30·9	32·9	37·3
0·3	32·5	28·9	30·4	30·8	38·1	34·6
1·0	29·8	29·3	31·4	30·4	33·0	36·6
3·0	29·6	29·6	31·0	34·0	34·3	36·0
Quillaia intake (mg/kg/day)						
Males						
0·3	260	120	90	80	100	70
1·0	780	360	310	290	320	280
3·0	2540	1200	960	810	838	700
Females						
0·3	280	170	130	110	100	90
1·0	930	540	460	350	330	370
3·0	2850	1540	1380	1140	1110	980

haemangiomas and haemangiosarcomas in the lymph nodes were similar in both control and treated animals. The one tumour showing a statistical difference from the control incidence was thyroid adenoma which occurred more frequently in females given 1% quillaia extract in the diet.

DISCUSSION

Many of the differences between the male rats given 3% quillaia extract and the control males may be accounted for in terms of the lower body weights recorded in the former group from wk 42. These include lower mortality, a higher concentrating ability in the renal function test at wk 78, together with a lower incidence of the more severe renal lesions, and decreased kidney, heart and thyroid weights.

It is known that animals on a reduced food intake show a prolonged lifespan (Gaunt, Hardy, Grasso *et al.* 1976; Ross & Bras, 1971; Simms, 1967). Moreover it has been established by other workers (Simms, 1967) and seen in this laboratory (Gaunt *et al.* 1976) that rats with a reduced body weight have a delayed onset of glomerulonephrosis. This was evident in the present study, since the incidence of animals with marked glomerulonephrosis was lower at the highest dietary level than in the controls. This difference could account for the lower water intake during the last 6 months of the study, the higher urinary specific gravity at wk 78 and the lower kidney weights. The urinary specific gravity of the treated rats at wk 78 was in fact similar to that recorded in both treated and control groups at earlier examinations, whereas the control value had decreased by wk 78. One result of nephrosis is left ventricular hypertrophy (Berg, 1967) and thus the delayed onset of the renal changes could account also for the lower heart weights in the treated rats. Snell (1967) showed that parathyroid hyperplasia occurs in animals with chronic nephrosis and since the parathyroid glands are included in the thyroid weights it is possible that a delay in this hyperplasia accounts for the differences recorded in the thyroid weights.

The lower body weights and associated findings in the rats fed 3% quillaia are ascribable to reduced food consumption, which was probably due to the unpalatability of the diet. The test of preference showed that diets containing quillaia extract were avoided and, in a 90-day experiment, Gaunt *et al.* (1974) found that the food intakes of rats given 2 or 4% quillaia extract were markedly reduced on the first day of treatment.

Table 4. *Mean renal concentration values following 16-hr water deprivation in rats fed diets containing 0–3% quillaia extract for 13, 24 or 78 wk*

Dietary level (%)	Urine values† At wk 13: Specific gravity	Volume (ml)	At wk 24: Specific gravity	Volume (ml)	At wk 78: Specific gravity	Volume (ml)
			Males			
0	1·078	0·1	1·072	0·2	1·055	1·1
3·0	1·063	0·1	1·075	0·2	1·075**	0·7
			Females			
0	1·084	0·1	1·065	0·2	1·067	0·7
3·0	1·082	0·1	1·090	0·1	1·078	0·2

†For 4-hr samples collected after a 16-hr period without water. Results are means for groups of ten rats. The value marked with asterisks differs significantly ($P < 0·01$: White, 1952) from the control value.

Tests for glucose, bile salts, blood and ketones were negative. Cell excretion rates, protein levels and microscopic constituents were similar in control and test samples.

Table 5. *Leucocyte counts in rats fed diets containing 0–3% quillaia extract for 15, 25, 52 or 108 wk*

Dose level (%)	No. of rats	Leucocytes: Total (10^3/mm^3)	Differential (%): N	E	L	M
Wk 15						
Males						
0	10	13·8	12	1	86	1
1	10	19·5*	—	—	—	—
3	10	23·0**	17**	1	80**	2
Females						
0	10	12·4	15	0	83	2
1	10	13·8	—	—	—	—
3	10	12·4	12	0	86	0
Wk 25						
Males						
0	10	13·8	21	1	78	0
1	10	12·2	—	—	—	—
3	10	18·5**	17	1	80	0
Females						
0	10	12·4	21	1	77	0
1	10	13·4	—	—	—	—
3	10	13·2	16	0	84	0
Wk 52						
Males						
0	10	14·1	20	0	77	1
1	10	13·8	—	—	—	—
3	10	14·8	21	0	76	2
Females						
0	10	10·2	15	1	82	1
1	10	10·3	—	—	—	—
3	10	10·3	12	1	86	1
Wk 108						
Males						
0	29	5·0	38	0	58	1
0·3	30	5·0	—	—	—	—
1	22	4·6	—	—	—	—
3	37	3·9*	37	1	61	0
Females						
0	31	3·3	36	0	62	0
0·3	33	3·5	—	—	—	—
1	37	3·0	—	—	—	—
3	34	2·6*	35	1	64	0

N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes

Values marked with asterisks differ significantly (Student's *t* test) from those for the corresponding controls: $*P < 0.05$; $**P < 0.01$. The arcsin transformation was used for percentage values. Basophils did not account for more than 0·5% of the leucocytes in any group.

a finding confirmed in the present study when 3% extract was given in the diet. These factors support the suggestion that the diet was unpalatable and the changes observed cannot be considered to be a sign of toxicity.

The observed significantly increased incidence of mortality in male animals given 1% extract in the diet during wk 87–91 was not accompanied by any consistent pathological changes either during that time or in the subsequent treatment period. Furthermore, female rats in either the 1 or 3% group showed no increased mortality. Therefore, this increase in male mortality is considered to be unrelated to treatment.

At the end of the study the total white cell counts of all groups including the controls were less than the earlier values. This is because the latter counts were performed on peripheral blood obtained from the tail veins whereas at wk 108 the blood was taken from the aorta. This difference has been observed in other studies (Evans, Butterworth, Gaunt & Grasso, 1977; Mason, Gaunt, Hardy *et al.* 1976). The differences in total leucocyte counts between the treated and control groups are not readily interpretable. The reduction in the total counts seen at wk 108 in animals of both sexes given 3% extract in the diet is probably a reflection of the decreased growth rate, as reported previously (Gaunt *et al.* 1976; Oishi, Oishi & Hiraga, 1979). The statistically significant increases at wk 15 and 25 were confined to the male animals and were not recorded at subsequent treatment periods. Furthermore, this finding at wk 15 is at variance with the results of an earlier 90-day study (Gaunt *et al.* 1974). In the light of these inconsistencies, these findings are not considered to be related to treatment.

The relative liver weights were lower in males fed 1% quillaia extract and higher in females given the 3% dietary level. This lack of consistency again supports the view that these findings cannot be attributed to treatment. The same comment can be made about the significant increases in weights of stomach, small intestine and caecum (full and empty) in the females since these occurred at the lowest and highest dietary levels, while the intermediate dose level showed no change from the controls, and no corresponding differences were observed in the males. Microscopic examination provided no evidence of irritation of the alimentary tract.

The slight increases in the incidence of heart fibrosis and dilatation of the glands of the gastric mucosa were evident only at the lowest treatment level in the females. This lack of any dose relationship, despite a ten-fold increase in the amount of test material given at the highest dietary level, and the lack of similar effects in the males, suggests that these are fortuitous variations of normal incidence rather than any effect of treatment.

It is relevant that the types of tumours seen in our treated rats have been reported elsewhere in untreated animals (Table 9). Furthermore no dose-related incidence of tumours was found in this study, and with one exception (the thyroid adenomas in the females fed 1% quillaia) there were no statistically significant increases in the numbers of any type of tumour compared with the control incidence. Even this exception was not dose-related, fewer being found at the higher dose level in the female rats while the incidence in all the treated male groups was lower than that in the controls. The total incidence of thyroid adenomas in both sexes fed 1% quillaia extract in their diet was not statistically different from the total control incidence. This evidence together with the known spontaneous occurrence of these tumours (Table 9) indicates that this minor increase was not related to quillaia treatment. The finding of haemangiomas and haemangiosarcomas of the lymph nodes was unusual, since these

Table 6. *Organ weights and relative organ weights of rats fed diets containing 0–3% quillaia extract for 2 yr*

Dietary level (%)	No. of rats examined	Organ													Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum		Adrenals†	Gonads‡	Pituitary†	Thyroid†	
									Full	Empty					
Organ weight (g)															
Males															
0	32	2·11	1·53	15·18	1·23	3·88	2·58	9·57	3·59	1·29	62	3 37	10·9	27·8	594
0·3	32	2·12	1·55	15·83	1·26	3·88	2·54	9·89	3·49	1·33	62	3·54	10·7	25·9	616
1·0	23	2·12	1·49	14·57	1·34	3·84	2·54	9·22	3·34	1·24	62	3·63	10·4	25·7*	633
3·0	39	2·10	1·44*	14·47	1·15	3·32*	2·41	9·69	3·49	1·37	57	3·55	10·7	23·5*	583
Females															
0	32	1·93	1·12	10·55	0·85	2·36	1·75	7·33	2·69	0·87	67	74	11·7	19·0	395
0 3	35	1·94	1·18	11·50	0·84	2·52	1·97*	8·15*	3·25*	1·09***	68	68	12·5	21·0	392
1·0	38	1·94	1·10	11·07	0·94	2·27	1·78	7·43	2·91	0·91	69	75	13·8	19·1	421
3·0	34	1·89	1·07	11·42	0·82	2·30	1·95	8·17**	3·45**	1·11***	61	68	12·7	21·2	377
Relative weight (g/100 g body weight)															
Males															
0	32	0·37	0·27	2·61	0·21	0·69	0·46	1·65	0·62	0·22	11	58·2	1·9	4·8	
0·3	32	0·36	0·26	2·56	0·21	0·66	0·43	1·68	0·58	0·22	10	59·6	1·8	4·4	
1·0	23	0·34	0·24	2·33*	0·21	0·62	0·41	1·48	0·54	0·20	10	58·1	1·7	4·2	
3·0	39	0·37	0·25	2·49	0·20	0·58	0·42	1·67	0·60	0·24	10	61·6	1·8	4·1	
Females															
0	32	0·51	0·30	2·71	0·22	0·62	0·46	1·92	0·70	0·23	18	19·0	3·0	5·0	
0·3	35	0·52	0·31	3·00	0·22	0·67	0·53	2·13	0·86*	0·28***	18	17·4	3·2	5·7	
1·0	38	0·48	0·27	2·66	0·23	0·56	0·43	1·80	0·72	0·22	17	18·3	3·3	4·6	
3·0	34	0·51	0·29	3·05*	0·22	0·63	0·54*	2·22**	0·94**	0·30***	16	18·0	3·4	5·6	

†Absolute and relative weights of this organ are expressed in mg and mg/100 g body weight, respectively.
‡Absolute and relative weights of female gonads are expressed in mg and mg/100 g body weight, respectively.
Values are the means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from the control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

Table 7. *Statistically significant histopathological findings (excluding tumours) in rats fed diets containing 0–3% quillaia extract for up to 2 yr*

Tissue and histological finding	Dietary level (%)...	Incidence of finding† Males 0	0·3	1·0	3·0	Females 0	0·3	1·0	3·0
Liver	*No. examined...*	*40*	*33*	*36*	*44*	*42*	*45*	*46*	*46*
Hepatocyte vacuolation and fatty change									
—mild/moderate		22	22	19	18	23	24	26	15*
—severe		3	2	2	3	4	7	3	1
Kidney	*No. examined...*	*39*	*36*	*38*	*42*	*42*	*45*	*46*	*44*
Glomerulonephrosis									
—mild/moderate		23	19	17	21	23	23	31	30
—severe		13	13	12	7	6	12	1	2
Calcium deposits		0	0	0	0	9	9	3*	4
Heart	*No. examined...*	*40*	*34*	*37*	*42*	*40*	*44*	*43*	*44*
Fibrosis		11	6	8	7	0	4*	2	2
Lung	*No. examined...*	*38*	*35*	*37*	*44*	*40*	*44*	*46*	*45*
Peribronchiole cuffing		0	0	0	0	6	6	4	1*
Spleen	*No. examined...*	*40*	*35*	*37*	*43*	*43*	*43*	*41*	*44*
Pigment		1	0	0	0	23	18	9**	18
Stomach	*No. examined...*	*40*	*33*	*37*	*43*	*42*	*42*	*44*	*44*
Glandular dilatation		1	4	0	1	2	9*	6	6
Parathyroid	*No. examined...*	*37*	*28*	*37*	*39*	*39*	*40*	*45*	*42*
Enlarged		5	3	4	3	8	5	1**	4
Vacuolated		0	0	1	0	1	0	0	0
Adrenal	*No. examined...*	*38*	*34*	*37*	*41*	*40*	*44*	*44*	*43*
Vascularization/haemorrhage		0	0	0	0	5	0*	0*	1
Harderian gland	*No. examined...*	*34*	*32*	*33*	*39*	*35*	*43*	*42*	*44*
Pigmentation		0	0	0	0	14	15	14	7*

†Incidence is expressed as the number of animals affected among the number examined (italicized). Values marked with asterisks differ significantly (chi-square test) from that for the corresponding control: $*P < 0.05$; $**P < 0.01$. No allowances were made for time of incidence.

Table 8. *Incidence of tumours in rats fed diets containing 0–3% quillaia extract for up to 2 yr*

Organ/tumour	Dietary level (%)...	No. of animals with tumours Males 0	0·3	1·0	3·0	Females 0	0·3	1·0	3·0
Liver	*No. examined...*	*40*	*33*	*26*	*44*	*42*	*45*	*46*	*46*
Small adenoma		0	0	0	0	0	0	0	1
Lung	*No. examined...*	*38*	*35*	*37*	*44*	*40*	*44*	*46*	*45*
Adenoma		0	0	0	0	0	1	0	0
Adenomatosis		0	0	0	0	0	1	0	0
Pancreas	*No. examined...*	*33*	*33*	*36*	*39*	*34*	*33*	*44*	*40*
Islet-cell adenoma		3	1	1	1	0	2	2	1
Adenocarcinoma (mucoid)		0	1†[a]	0	0	0	1	0	0
Exocrine adenoma		1	0	1	1	0	0	0	0
Pituitary	*No. examined...*	*30*	*29*	*34*	*35*	*36*	*39*	*40*	*42*
Adenoma		8	9	7	6	23	21	22	18
Carcinoma		0	0	0	1	0	0	1	3†[b]
Ileum	*No. examined...*	*39*	*32*	*35*	*42*	*41*	*44*	*44*	*45*
Adenocarcinoma		0	1	0	1	0	0	0	0
Leiomyoma		1	0	0	0	0	0	0	0
Other tumour		0	0	0	0	0	0	0	1
Adrenal	*No. examined...*	*38*	*34*	*37*	*41*	*40*	*44*	*44*	*43*
Medullary adenoma		0	1	0	3	0	0	0	1
Medullary carcinoma		0	0	1	1	0	0	0	0
Phaeochromocytoma		1	1	0	0	0	0	0	0
Cortical adenoma		0	0	0	1	0	0	0	0
Thyroid	*No. examined...*	*37*	*28*	*37*	*39*	*39*	*40*	*45*	*42*
Adenoma		5	3	3	3	0	2	5*	4
Carcinoma		1	0	1	0	0	0	1	0
Parathyroid adenoma		0	0	0	0	0	1	0	0

[*contd*]

Table 8 (*continued*)

Organ/tumour	Dietary level (%)...	No. of animals with tumours							
		Males				Females			
		0	0·3	1·0	3·0	0	0·3	1·0	3·0
Skin, subcutaneous tissue and mammary gland‡	*No. examined...*	*40*	*37*	*38*	*44*	*43*	*45*	*46*	*46*
Haemangioma		0	1	1	0	0	0	0	0
Fibroma		3	2	2	4	1	0	3	4
Fibrosarcoma		0	0	2	0	1	0	0	1
Lipoma		0	1	0	0	0	0	0	1
Adenocarcinoma		0	0	0	0	3	0	2	2
Osteosarcoma		1	0	0	0	0	0	0	0
Reticulum-cell sarcoma		0	0	1†[c]	0	0	0	0	0
Fibroadenoma/adenoma		0	0	1	0	15	14	8	11
Carcinoma—undifferentiated		0	0	0	0	0	0	2	0
—basal-cell		0	0	0	0	0	1	0	0
—squamous-cell		0	0	0	0	1	0	1	0
Thymus	*No. examined...*	*4*	*16*	*18*	*18*	*5*	*8*	*12*	*11*
Thymoma		1	0	0	0	0	0	0	0
Lymphoma		0	0	0	0	0	0	1	0
Lymphosarcoma		0	0	0	0	1	1	0	1
Harderian gland	*No. examined...*	*34*	*33*	*32*	*39*	*35*	*33*	*42*	*44*
Carcinoma		0	0	1†[c]	0	0	0	0	0
Uterus	*No. examined...*					*34*	*44*	*41*	*43*
Adenocarcinoma		—	—	—	—	0	2	1	1
Leiomyoma		—	—	—	—	0	0	1	0
Haemangioma		—	—	—	—	0	1	0	0
Testis	*No. examined...*	*37*	*34*	*36*	*43*				
Interstitial-cell tumour		1	3	0	0	—	—	—	—
Kidney	*No. examined...*	*39*	*36*	*38*	*42*	*42*	*45*	*46*	*44*
Lipoma		0	0	0	0	0	0	1	0
Leiomyosarcoma		1	0	0	0	0	0	0	0
Nephroblastoma		0	0	0	0	1	0	0	0
Peritoneal cavity‡	*No. examined...*	*44*	*37*	*38*	*44*	*43*	*45*	*46*	*46*
Fibrosarcoma		1	0	0	0	0	0	0	0
Other sarcomas		0	1	0	0	0	0	1	1†[d]
Salivary gland	*No. examined...*	*40*	*35*	*34*	*40*	*42*	*43*	*45*	*42*
Adenoma		0	0	0	0	1	0	0	0
Brain	*No. examined...*	*40*	*35*	*38*	*43*	*41*	*43*	*46*	*45*
Astrocytoma		0	0	1	0	0	0	0	0
Other tumour		0	0	0	0	1	0	0	0
Lymph node	*No. examined...*	*33*	*29*	*32*	*38*	*35*	*41*	*41*	*36*
Haemangioma		1	2	1	1	1	0	1	2
Haemangiosarcoma		1	1	0	0	0	0	0	0
Lymphoma		0	2	0	0	0	0	0	0
General									
Lymphosarcoma		0	0	0	1†[c]	0	0	0	0

†Secondary deposits—(a) in salivary gland, adrenal gland, lymph node, heart, lung and Harderian gland; (b) invading brain, in one rat; (c) in lung; (d) in spleen; (e) in lung, liver, spleen and kidney.

‡Lesions identified at autopsy in the number of animals shown.

The figure marked with an asterisk differs significantly ($P < 0.05$ by chi-square test) from the corresponding control value. No allowances were made for the time of incidence.

tumours have occurred rarely in previous studies. However, the incidence of these tumours was noted in both control and treated animals.

This study, in which quillaia extract was fed at levels up to 3% in the diet for 2 yr, confirmed the results of the previous study in mice (Phillips *et al.* 1979) which failed to detect any carcinogenic effects. The no-untoward-effect level established in this rat study was 3% in the diet, which is equivalent to an intake of approximately 1·5 g/kg/day. This is considerably in excess of the possible intake from the maximum permitted level of quillaia extract (200 ppm) in soft drinks. The difference between the no-untoward-effect level in the present study and that of 0·7 g/kg/day obtained in the long-term study in mice (Phillips *et al.* 1979) suggests a species difference, while the wide difference between this result in rats and the no-untoward-effect level of 0·4 g/kg/day deduced from the short-term rat study (Gaunt *et al.* 1974) was probably due to the rats' becoming accustomed to the quillaia extract over the prolonged feeding period.

Table 9. *Literature references to the spontaneous occurrence of tumours in the rat*

Organ	Tumour	References*
Thyroid	Adenocarcinoma	1, 5, 7
Pancreas	Adenocarcinoma	1, 5, 7
Adrenal	Medullary-cell types	5, 6
Lung	Adenocarcinoma	1, 3
Salivary gland	Various types	1, 4, 5, 7
Uterus	Adenocarcinoma	1, 4, 5
	Leiomyoma	2
Peritoneum	Reticulum-cell sarcoma	2
Brain	Astrocytoma	4
Lymph nodes	Sarcoma	5, 6
Subcutaneous tissue	Lipoma	6

*(1) Brantom, Gaunt, Hardy *et al.* (1973); (2) Evans *et al.* (1977); (3) Gaunt, Brantom, Grasso *et al.* (1972); (4) Gaunt, Carpanini, Grasso & Lansdown (1972); (5) Gaunt, Butterworth, Hardy & Gangolli (1975); (6) Gaunt *et al.* (1976); (7) Snell (1965).

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CARCINOGENICITY STUDIES OF SODIUM NITRITE AND SODIUM NITRATE IN F-344 RATS*

A. Maekawa, T. Ogiu, H. Onodera, K. Furuta,
C. Matsuoka, Y. Ohno and the late S. Odashima

Division of Pathology, Biological Safety Research Centre, National Institute of Hygienic Sciences, Kamiyooga 1-18-1, Setagaya-ku, Tokyo 158, Japan

(*Received 25 March 1981*)

Abstract—The carcinogenicity of sodium nitrite and of sodium nitrate was examined in F-344 rats. Sodium nitrite was administered in the drinking-water for 2 yr at levels of 0·125 or 0·25%. Sodium nitrate was given in the diet at levels of 2·5 or 5%. A variety of tumours occurred in all groups including the controls. The only significant difference between treated and control groups in the total number of tumours detected in either of the studies was a significant decrease in tumour incidence in the high-dose females given nitrite compared with controls. There was no positive dose-response relationship either in the incidence or in the induction time of tumours in either of the studies. The only significant result was a reduction in the incidence of mononuclear cell leukaemias in the experimental groups in both studies. It is concluded that sodium nitrite and sodium nitrate did not exert a carcinogenic effect that could be detected under the conditions of this study in which the animals showed a high incidence of spontaneous tumours.

INTRODUCTION

Sodium nitrite and sodium nitrate are widely used in Japan and throughout the world as food additives to preserve and colour cured meat and/or fish. It was recently reported that sodium nitrite has strong mutagenic activity in various mutagenicity tests (Odashima, 1980). It has also been demonstrated that sodium nitrite is a precursor of *N*-nitroso compounds, many of which have strong carcinogenic activity in many species of animals. Sodium nitrate is partially reduced to sodium nitrite both in the animal body and in food (Heisler, Siciliano, Krulick *et al.* 1974; Ishiwata, Boriboon, Nakamura *et al.* 1975). But until now there have been no significant reports on the carcinogenicity test of these two chemicals. The present studies were carried out to clarify the carcinogenicity of these two chemicals.

EXPERIMENTAL

Subchronic toxicity study

A total of 240 Fischer-344 rats (SPF, 5-wk-old) of both sexes were purchased from Charles River Japan Inc. (Kanagawa). Rats were housed four to a plastics cage and kept in an air-conditioned animal room (temperature 25 ± 2°C, humidity 55 ± 10%).

Sodium nitrite (special grade reagent, purity 98·5%) and sodium nitrate (special grade reagent, purity 99·5%) were purchased from Koso Chemical Co. Ltd (Tokyo). The stability of the sodium nitrite when mixed with the diet was found to be very low (13–27% recovery) and it was therefore administered in the drinking-water. A fresh solution of sodium nitrite in distilled water was prepared daily. Sodium nitrate was found to be stable in the diet (about 100% recovery) and was therefore mixed with the basic diet (CRF-1, Charles River Japan Inc.) which was then pelleted.

The animals were divided into groups of ten male and ten female rats. In the sodium nitrate study five experimental groups were given as drinking-water 20 ml of a solution of sodium nitrite/rat/day at concentrations of 1, 0·5, 0·25, 0·125 or 0·06% for 6 wk. Rats in the control group were each given 20 ml distilled water/day. In the sodium nitrate study the five treated groups were given 20, 10, 5, 2·5 or 1·25% sodium nitrate in the diet *ad lib.* for 6 wk. The control group was given basic diet only. In the nitrate study all groups were given tap water freely.

During the experimental periods, all the animals were observed daily; signs of toxicity and mortality were recorded, and body weights were determined every other week. Dead animals were completely autopsied. At the end of the study, all surviving animals were killed for gross and microscopical examinations. The results of this study were used to determine the appropriate dose-levels in the carcinogenicity study.

Carcinogenicity study

Rats. A total of 600 F-344 rats of both sexes (SPF, 5-wk-old), purchased from Charles River Japan Inc., were maintained on the basic diet (CRF-1) and tap water, until they were 8-wk-old when the studies were started. Animals were divided into groups comprising 50 male and 50 female rats.

Housing and feeding conditions. Rats were housed four males and five females to a plastics cage and kept in an air-conditional animal room (conventional animal room without barrier system) at a temperature

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Abbreviations: NDMA = *N*-Nitrosodimethylamine; SPF = specific-pathogen free.

25 ± 2 C and a relative humidity of 55 ± 10%. The basic diet was analysed for contaminants twice a year and it was ascertained that contaminants such as pesticides, metals, benzopyrene and aflatoxin were not included in the diet. Sodium nitrite and sodium nitrate were as used in the subchronic toxicity study.

Experimental design. In the nitrite study, the experimental groups were given 20 ml 0·25% (maximum tolerated dose) or 0·125% sodium nitrite/rat/day as their drinking water for 2 yr. Rats in the control group were each given 20 ml distilled-water/day for 2 yr. In the nitrate study the experimental groups were given diets containing 5% (maximum tolerated dose) or 2·5% of sodium nitrate *ad lib.* for 2 yr. Rats in the control group were given basic diet without nitrate *ad lib.* for 2 yr. The stability of nitrate in the diet was determined whenever new diet was received, and it was ascertained that the recovery rate of the chemical was about 100% at all times. Administration of chemicals was stopped at wk 104 and thereafter tap water and basic diet was given in all experimental groups, and observation was continued until wk 120 in the nitrite study and wk 123 in the nitrate study when all survivors were killed. These times were selected because the number of survivors in at least one group of either sex was less than 10 (20%). During the experimental period all animals were observed daily, and clinical signs and mortality were recorded. The amount of nitrite-containing water consumed/day or the amount of nitrate-containing diet consumed/month was measured. Body weights were recorded once a week during the first 10 wk of the study and every 2 wk thereafter. Moribund or dead animals were autopsied completely and examined for the development of tumours in various organs and/or tissues. Organs and/or tissues were fixed with buffered 10% formalin, and sections were stained routinely with haematoxylin and eosin.

Analysis of nitrosamines. Analysis of nitrosamines in the basic diet, diet containing nitrate, drinking-water containing nitrite and the stomach contents of treated rats was carried out twice using a Thermo-Energy Analyser (TEA-502), Shimazu Inc., Japan). For analysis of *N*-nitrosamines in the stomach contents, groups of ten male and ten female 10-wk-old F-344 rats were given identical treatments to the animals in the main study. After 1 wk of treatment the animals were killed and their stomach contents were analysed.

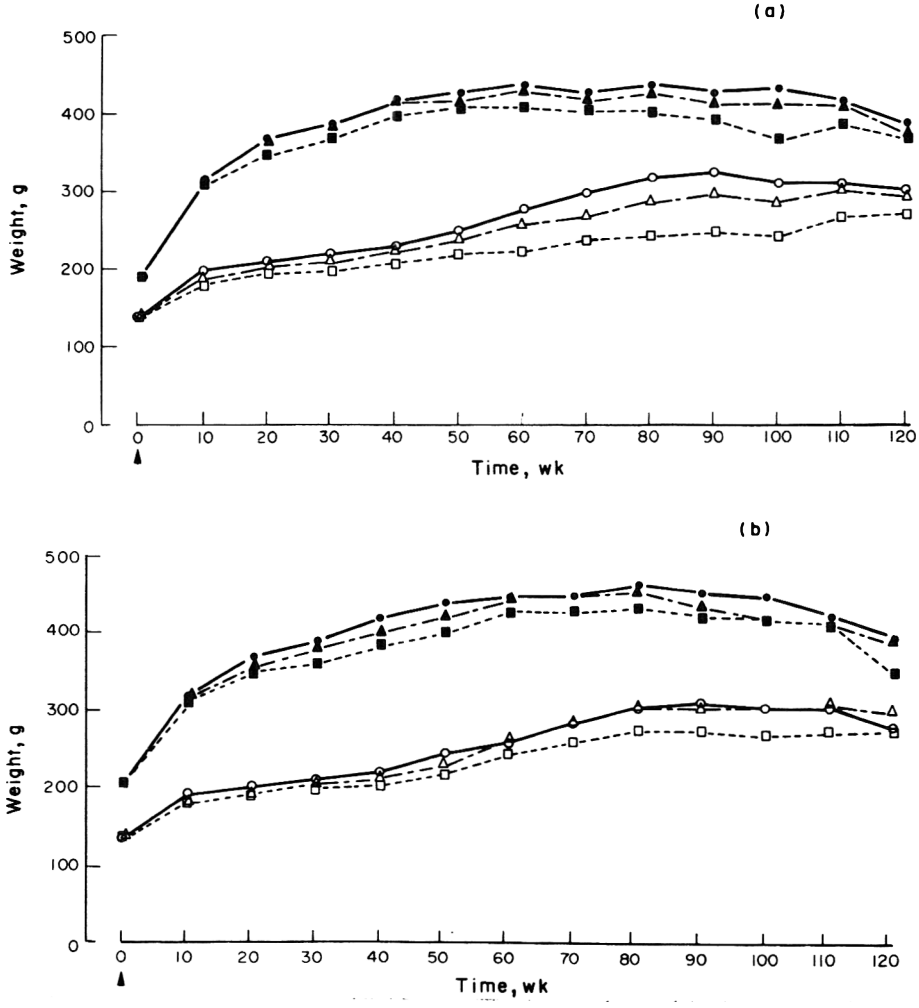

Fig. 1. Growth curves of rats treated with (a) sodium nitrite in the drinking water and (b) sodium nitrate in the diet. Control males (—●—), low-dose males (-—▲—-), high-dose males (---■---), control females (—○—), low-dose females (-—△—-) and high-dose females (---□---).

Table 1. *Cumulative mortality of rats treated with sodium nitrite in the drinking-water or sodium nitrate in the diet*

Sex	Dose†	Cumulative mortality (%) at 80 wk	100 wk	Term‡
	Sodium nitrite study			
M	0 (control)	16	32	76
	0·125	6	30	52*
	0·25	4*	12*	42**
F	0 (control)	2	14	54
	0·125	4	14	46
	0·25	8	16	48
	Sodium nitrate study			
M	0 (control)	6	20	80
	2·5	0	8	52**
	5·0	8	24	62*
F	0 (control)	12	36	72
	2·5	6	20	46**
	5·0	12	24	50*

†The dose is expressed as the percentage in the drinking-water (sodium nitrite) or diet (sodium nitrate).
‡In the sodium nitrite study, 120 wk; in the sodium nitrate study, 123 wk.
Values marked with asterisks differ significantly (chi-square test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$).

RESULTS

Subchronic toxicity study

During the 6 wk of sodium nitrite treatment four female rats in the 1% group, and one male and one female in the 0·5% group died. None of the other treated or control rats died. In all the experimental groups except the 1% group, depression of body-weight gain compared with the control group was less than 10%. In the case of nitrate, all the females and seven males in the 20% group died during the experiment. In all groups except the males given 20% and the females given 10% nitrate, the decrease of body-weight gain compared with controls was less than 10%.

At autopsy the abnormal colour of the blood and the spleen due to methaemoglobin was marked in rats of the two highest dose groups in both studies.

From these results it was determined that the maximum tolerated doses of sodium nitrite and nitrate in F-344 rats were 0·25% in drinking-water and 5% in the diet, respectively.

Carcinogenicity studies of nitrite and nitrate

The first rats with tumours were autopsied at wk 56 in the sodium nitrite study and at wk 36 in nitrate study. Rats that survived beyond wk 56 and wk 36 in the nitrite and nitrate studies respectively were included in the data. Figure 1 shows the growth curve of the animals in both studies. In the male high-dose groups the mean body weight differed from that of the control group by less than 10% at all times in both studies, but in the female high-dose groups mean body weights were reduced by more than 10% after wk 40 in the nitrite study and after wk 60 in the nitrate study. Table 1 demonstrates the cumulative mortality of rats in the two studies. At term both male and female control groups contained the lowest numbers of survivors in both studies.

The amount of nitrite or nitrate consumed, the total tumour incidence including both benign and malignant tumours and the mean survival times are given in Table 2. Daily consumption of water or diet was almost constant throughout the experimental period in all groups. These consumption data were used to calculate the nitrite and nitrate intake data given in Table 2. The incidence of tumours was 100% in all male groups in the nitrite study and nearly 100% in all male groups in the nitrate study. However there was a reverse dose-effect relationship in the incidence of tumours in the females of both studies. The tumour incidence in the high-dose group was signifi-

Table 2. *Amount of nitrite or nitrate consumed, tumour incidence and mean survival time of F-344 rats after continuous oral administration of sodium nitrite or nitrate*

Dose (%)†	Sex	Effective no. of rats‡	Total nitrite or nitrate consumed (g/rat)	No. of rats with tumours (%)	Survival time (wk) Mean ± SD	Range
			Sodium nitrite			
0 (control)	M	46	0	46 (100)	108 ± 13·6	72–120
	F	49	0	45 (92)	113 ± 10·6	82–120
0·125	M	49	19	49 (100)	109 ± 13·9	72–120
	F	48	15	41 (85)	114 ± 9·8	84–120
0·25	M	50	34	50 (100)	113 ± 13·2	56–120
	F	48	25	35 (73)*	112 ± 12·7	58–120
			Sodium nitrate			
0 (control)	M	50	0	47 (94)	108 ± 16·8	44–123
	F	50	0	46 (92)	105 ± 17·4	63–123
2·5	M	50	277	50 (100)	116 ± 9·3	90–123
	F	50	190	43 (86)	113 ± 15·6	68–123
5·0	M	50	575	48 (96)	109 ± 20·1	36–123
	F	49	394	39 (80)	109 ± 20·7	39–123

†The dose is expressed as the percentage in the drinking-water (sodium nitrite) or the percentage in the diet (sodium nitrate).
‡Initially each group comprised 50 animals.
The value marked with an asterisk differs significantly ($P < 0.05$; chi-square test) from the corresponding control value.

cantly decreased ($P < 0.05$) compared with that in the control group in the nitrite study, but there were no significant differences in the nitrate study. There was no significant difference in mean survival times between control and experimental groups in either study.

Tables 3 and 4 show the mean incidences and induction times of tumours of various organs and/or tissues. As shown in the tables, in all groups of males in both studies, tumours of the testis were the most frequent, followed by those of the mammary gland, adrenal gland and liver. Tumours of the mammary gland, pituitary gland, uterus, and adrenal gland were those detected most frequently in the female rats of both studies. Tumours were also detected in other organs and/or tissues of each group of both studies, although the incidence was relatively low. None of the treatment groups showed a significant increase in the incidence of any specific tumour compared with the corresponding control group. There was also no significant difference between experimental and control groups in the mean induction time of any tumour.

The only interesting difference between treated animals and controls is in the incidence of tumours of the haematopoietic organs in both studies. The incidence of these tumours was relatively high in the control groups of both sexes in both studies. The incidence was, however, significantly decreased in experimental groups, especially in the nitrate study.

Table 5 shows the age-related incidence of tumours of the testis, uterus, haematopoietic organs, pituitary gland, adrenal gland, liver and thyroid gland. These tumours increased rapidly after about 2 yr in each group of both studies and there was no particular tumour which seemed to appear earlier in each experimental group.

The tumours found in the two studies were histologically similar. All the testicular tumours that were detected were interstitial cell tumours. The mammary tumours differed histologically in male and female rats. In the male rats the epithelial element of the fibroadenoma was strongly atrophic and only the mesenchymal element proliferated as if it was a fibroma whereas typical fibroadenomas were the most frequent mammary tumours in the females. Most of the pituitary tumours were chromophobic adenomas. The tumours observed in the haematopoietic organs were mononuclear cell leukaemias, except for one other tumour in a male given the lower dose of nitrite. In cases of mononuclear cell leukaemia, clinical signs such as severe emaciation, anaemia, jaundice and abdominal distension were observed and at autopsy splenomegaly or hepatosplenomegaly was marked. Enlargement of lymph nodes was not so marked, although in a few cases systemic enlargement of lymph nodes was detected. Atypical mononuclear cells like monocytes or lymphocytes were detected in peripheral blood and in many cases erythrophagia was observed. Histochemical tests for peroxidase were negative. Invasion of leukaemic cells was observed in the spleen and liver with few exceptions and in some cases tumour-cell invasion was also observed in lymph nodes, lungs, kidneys, adrenal glands and bone marrow. Most adrenal tumours were small phaeochromocytomas, although there were a few cases of phaeochromocytomas combined with ganglioneuromas, and large malignant phaeochromocytomas with metastases in remote organs; cortical tumours were very rare. Most liver tumours were benign adenomas, although a few hepatocellular carcinomas were also observed. In the thyroid gland, C-cell adenomas were most common, although other types of thyroid tumours such as papillary adenomas and C-cell carcinomas were also observed. Endometrial angiomatous polyps, adenomas and adenocarcinomas were frequent in the uterus. The histology of the other tumours detected was similar to that of the spontaneous tumours reported by other investigators (Moloney, Boschetti & King, 1970; Sass, Rabstein, Madison *et al.* 1975).

There was no significant difference in the incidence of malignant tumours between experimental and control groups of both studies.

Table 6 shows the results of analysis for *N*-nitrosamines of the basic diet, diet containing nitrate, drinking-water containing nitrite, and the stomach contents of treated rats. More NDMA was detected in the nitrate-containing diet than in the basic diet. The maximum value, 37 ppb NDMA, was detected in the stomach contents of rats given sodium nitrite in their drinking-water and up to 11 ppb was found in the stomach contents of rats given nitrate in the diet, although no nitrosamine was detected in the stomach contents of control rats. No other *N*-nitroso compounds were detected.

DISCUSSION

Druckrey, Steinhoff, Buethner *et al.* (1963) reported that sodium nitrite was not carcinogenic in rats when it was given continuously in the drinking-water for their life-span. It has since been suggested that sodium nitrite is mutagenic in various mutagenicity tests (Odashima, 1980). Inai, Aoki & Tokuoka (1979) recently reported a carcinogenicity test in ICR mice in which sodium nitrite was given at levels of 0·5, 0·25 and 0·125% in the drinking-water for 109 wk and carcinogenicity was not detected. On the other hand, Newberne (1979) reported that sodium nitrite promoted lymphomas in experimental groups when Sprague–Dawley rats were given 250–2000 ppm of sodium nitrite in the diet or drinking-water but Newberne's data have since been invalidated (Dickson, 1980). In addition, Inui also reported transplacental action of nitrite on hamster embryo cells (Inui, Nishi, Taketomi & Mori, 1979). Most recently, Mirvish, Bulay, Runge & Patil (1980) reported that in MCR Wistar rats receiving sodium nitrite in drinking-water (3 g/litre) 8 of 45 rats (18%) had papillomas of the forestomach and the incidence was significantly greater than that in the control group (2%). However, there have been no significant reports on the carcinogenicity of sodium nitrate, although Sugiyama, Tanaka & Mori (1979) reported recently that sodium nitrate had been found to be non-carcinogenic in ICR mice.

In our studies many different types of tumours were observed in all groups including the controls. The distribution and histology of those tumours were similar to those of the spontaneous tumours mentioned by other investigators (Moloney *et al.* 1970; Sass *et al.* 1975). In our studies the incidence of spontaneous

Table 3. *Organ distribution and first time of detection of tumours in F-344 rats treated with ...*

Treatment group...	Control		0·125%		0·25%	
Affected organ or tissue	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)
			Males			
Testis	46 (100)	72	46 (94)	72	48 (96)	80
Mammary gland	9 (20)	91	15 (31)	91	13 (26)	108
Pituitary gland	5 (11)	72	5 (10)	88	3 (6)	80
Haematopoietic organs	16 (35)	72	5 (10)*	94	5 (10)*	105
Adrenal gland	9 (20)	98	11 (22)	94	5 (10)	118
Liver	4 (9)	115	5 (10)	98	7 (14)	119
Thyroid gland	4 (9)	102	4 (8)	97	0	
Peritoneum	1 (2)		4 (8)	87	2 (4)	101
Nervous system	1 (2)		1 (2)	120	1 (2)	56
Subcutaneous tissue	9 (20)	80	4 (8)	72	3 (6)	116
Lung	2 (4)	111	3 (6)	111	2 (4)	117
Preputial gland	3 (7)	84	3 (6)	85	7 (14)	84
Pancreas	3 (7)	91	5 (10)	111	4 (8)	108
Mediastinum	1 (2)	113	0		0	
Tongue	1 (2)	98	0		0	
Spleen	1 (2)	120	3 (6)	84	0	
Forestomach	1 (2)	103	0		0	
Skin	1 (2)	120	0		1 (2)	117
Heart	0		1 (2)	120	0	
Ear duct	0		0		1 (2)	101
Small intestine	0		0		1 (2)	120
Large intestine, rectum	0		0		2 (4)	88
Urinary bladder	0		1 (2)	120	0	
Kidney	0		0		2 (4)	119
Prostate	0		1 (2)	97	0	
			Females†			
Mammary gland	24 (49)	89	22 (46)	110	14 (29)*	80
Pituitary gland	17 (35)	86	11 (23)	101	14 (29)	80
Haematopoietic organs	13 (27)	82	7 (15)	95	4 (8)*	102
Adrenal gland	2 (4)	112	3 (6)	112	5 (10)	101
Liver	1 (2)	120	0		0	
Thyroid gland	4 (8)	100	3 (6)	120	3 (6)	101
Uterus	9 (18)	100	10 (21)	85	4 (8)	98
Nervous system	1 (2)	115	0		1 (2)	58
Subcutaneous tissue	0		1 (2)	120	2 (4)	118
Lung	3 (6)	116	3 (6)	120	1 (2)	120
Clitoral gland	0		1 (2)	114	4 (8)	101
Pancreas	2 (4)	89	1 (2)	120	0	
Forestomach	0		1 (2)	85	0	
Small intestine	0		1 (2)	109	1 (2)	120
Urinary bladder	0		1 (2)	112	0	

†Among females there were no tumours of the peritoneum, mediastinum, tongue, spleen, skin, heart, large intestine, rectum or kidney.
Values marked with asterisks differ significantly ($P < 0.05$; chi-square test) from the corresponding control values.

Table 4. *Organ distribution and first time of detection of tumours in F-344 rats treated with sodium nitrate*

Treatment group ...	Control		2·5%		5·0%	
Affected organ or tissue	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)
Males						
Testis	44 (88)	78	49 (98)	90	39 (78)	98
Mammary gland	17 (34)	83	9 (18)	111	11 (22)	102
Pituitary gland	3 (6)	106	3 (6)	102	4 (8)	87
Haematopoietic organs	18 (36)	78	2 (4)*	90	1 (2)*	
Adrenal gland	9 (18)	101	14 (28)	101	12 (24)	98
Liver	6 (12)	106	7 (14)	116	4 (8)	101
Thyroid	1 (2)	78	6 (12)	108	6 (12)	98
Peritoneum	2 (4)	109	3 (6)	90	2 (4)	107
Nervous system	1 (2)	120	3 (6)	116	2 (4)	36
Subcutaneous tissue	2 (4)	44	5 (10)	92	3 (6)	100
Lung	2 (4)	120	2 (4)	116	3 (6)	78
Preputial gland	5 (10)	94	2 (4)	123	2 (4)	100
Pancreas	2 (4)	106	5 (10)	122	6 (13)	88
Tongue	1 (2)	113	0		0	
Spleen	0		3 (6)	113	0	
Skin	0		1 (2)	123	0	
Heart	0		1 (2)	123	0	
Ear duct	1 (2)	90	0		0	
Small intestine	0		0		1 (2)	100
Large intestine, rectum	0		0		2 (4)	101
Salivary gland	0		1 (2%)	108	0	
Females†						
Mammary gland	20 (40)	75	20 (40)	104	13 (27)	68
Pituitary gland	17 (34)	89	18 (36)	74	11 (22)	85
Haematopoietic organs	14 (28)	74	0*		1 (2)*	119
Adrenal gland	4 (8)	70	6 (12)	79	4 (8)	115
Liver	2 (4)	63	0		0	
Thyroid gland	2 (4)	91	0		1 (2)	123
Uterus	4 (8)	70	10 (20)	74	6 (12)	98
Nervous system	0		2 (4)	87	1 (2)	45
Subcutaneous tissue	2 (4)	103	4 (8)	89	1 (2)	95
Lung	0		2 (4)	123	2 (4)	123
Clitoral gland	3 (6)	123	3 (6)	68	6 (12)	68
Pancreas	0		1 (2)	123	1 (2)	123
Spleen	0		0		1 (2)	115
Skin	0		1 (2)	121	0	
Ear duct	1 (2)	93	1 (2)	93	0	
Small intestine	0		0		1 (2)	109
Ovary	1 (2)	95	1 (2)	123	0	
Salivary gland	1 (2)	123	0		0	

†Among females there were no tumours of the peritoneum, tongue, heart, large intestine or rectum.
Values marked with asterisks differ significantly ($P < 0.01$; chi-square test) from the corresponding control values.

Table 5. *Age-related incidence of tumours in F-344 rats treated with sodium nitrite or sodium nitrate*

Sex	Age group (months)	Treatment group...	No. of rats with tumours of the: Testis or uterus C	L	H	Mammary gland C	L	H	Haemato-poietic organs C	L	H	Pituitary gland C	L	H	Adrenal gland C	L	H	Liver C	L	H	Thyroid C	L	H
Sodium nitrite study																							
Male	13–15																						
	16–18		2	1					1			1											
	19–21		3	4	3								1	1									
	22–24		11	7	3	3	1		4	1		1	1		4	1			1		1	1	
	25–27		13	8	10	3	2	5	6	3	4	1	2	1	1	2		1	1			1	
	28–		17	26	32	3	22	9	5	1	1	2	1	1	4	8	5	3	3	7	3	2	
Female	13–15																						
	16–18																						
	19–21		1	1		1		1	2	1		2		2									
	22–24		1	1	2		1	2	1	1	1	2	1	1			2				1		1
	25–27		5	2		10	3	1	6	3	3	3	4	4	1	1	1				1		
	28–		2	6	2	13	18	10	4	2		10	6	7	1	2	2	1			2	3	2
Sodium nitrate study																							
Male	13–15																						
	16–18		1						1												1		
	19–21		1	1		2			1	1				1									
	22–24		10	5	7	4		2	4				1	1	2		3			1			2
	25–27		13	12	5	3	3	1	9	1		1			3	6	2	1	1			2	
	28–		19	31	27	8	6	8	3		1	2	2	2	4	8	7	5	6	3		4	4
Female	13–15																	1					
	16–18		2	1		1		1	1				1		2								
	19–21					1		1	1			1	3	1		1		1					
	22–24		2		1	5	1	3	6			7	2	2							2		
	25–27			1		5	1	2	3			3	2	2	1								
	28–			8	5	8	18	6	3		1	6	10	6	1	5	4						1

C = Control group L = Low-dose group H = High-dose group

Table 6. *Determination of N-nitroso compounds in the diets, drinking-water and stomach contents of nitrite- and nitrate-treated and control rats*

Material analysed	NDMA (ppb)	
Basic diet (CRF-1)	7·5	ND
Diet containing 2·5% nitrate	14·4	49·2
Diet containing 5% nitrate	12·9	27·5
Water containing 0·125% nitrite	ND	ND
Water containing 0·25% nitrite	ND	ND
Stomach contents of control males	ND	ND
Stomach contents of control females	ND	ND
Stomach contents of 2·5% nitrate males	Trace	
Stomach contents of 2·5% nitrate females	10·7	
Stomach contents of 5% nitrate males	10·1	6·8
Stomach contents of 5% nitrate females	10·0	10·8
Stomach contents of 0·125% nitrite males	Trace	
Stomach contents of 0·125% nitrite females	9·0	
Stomach contents of 0·25% nitrite males	36·8	18·0
Stomach contents of 0·25% nitrite females	13·5	15·2

ND = Not detected NDMA = *N*-Nitrosodimethylamine
Nitrosamines other than NDMA were not detected.

tumours in the controls was very high. Comparing the total incidence of tumours in treated and control groups is therefore of little value. None of the tumour types had a significantly higher incidence among treated animals than among controls. The age-related tumour incidences also demonstrated a lack of effect of the treatments.

The most interesting result in the intergroup difference in the incidence of leukaemias. The incidence of mononuclear cell leukaemias was relatively high in all the control groups. F-344 rats have a high incidence of spontaneous mononuclear cell leukaemias. The spontaneous incidence has been reported as 31% in males and 21% in female rats (Sass *et al.* 1975) and as about 25% (Moloney, 1970). The incidence of the leukaemias in the controls in our studies was about the same or slightly above these previous reports but the incidence in experimental groups was much lower. The reason for this reduction in the incidence of spontaneous leukaemias is not clear but in treated rats there was slight atrophy of the haematopoietic organs such as the spleen and lymph nodes. This finding may be important in the reduction of leukaemias.

Our results were widely different from those of Newberne (1979) but as previously mentioned Newberne's data have been invalidated. Mirvish *et al.* (1980) reported that sodium nitrite induced papillomas of the forestomach in Wistar rats but in our sodium nitrite study only one female rat in the 0·125% group developed forestomach papillomas.

Sodium nitrite is a precursor of *N*-nitroso compounds and in combination with secondary amines can form nitrosamines in food or in the body. Sodium nitrate is also a precursor of *N*-nitroso compounds since it can be reduced to the nitrite either in the body or in food (Heisler *et al.* 1974; Ishiwata *et al.* 1975).

Aoyagi, Matsukura, Uchida *et al.* (1980) recently reported induction of liver tumours in Wistar rats by sodium nitrite given in pelleted diet. Volatile *N*-nitroso compounds, especially NDMA were detected at ppm levels in the pelleted diet used and they discussed the possibility that *N*-nitroso compounds formed in the treated diets were the main cause of liver tumours.

In our studies, there was more NDMA in the diet containing sodium nitrate than in the basic diet, and NDMA was detected in the stomach contents of rats given sodium nitrite or nitrate, although no nitrosamine was detected in the stomach contents of control animals. But the amount of NDMA detected in our studies was very low compared with the minimum carcinogenic dose reported by Terracini, Magee & Barnes (1967). It is well known that the main target organs of NDMA are the liver and the kidney in rats. In our studies the incidence of liver tumours in experimental groups was no higher than that among the controls and there was also no treatment-related effect on the incidence of kidney tumours. These results suggest that the tumours including liver tumours observed in our studies were not due to NDMA.

It is concluded that sodium nitrite and sodium nitrate did not have carcinogenic activity in F-344 rats when they were administered continuously in the drinking water or diet for 2 yr. It is, however, very important to reduce the level of these chemicals in foods, because it is known that they are precursors of the carcinogenic *N*-nitroso compounds.

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FURTHER INVESTIGATIONS OF MUTAGENIC CHOLESTEROL PREPARATIONS

G. A. S. ANSARI, R. D. WALKER, V. B. SMART and L. L. SMITH
Division of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550, USA

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Abstract—The previously demonstrated mutagenicity of naturally air-aged (autoxidized) USP cholesterol in test strains of *Salmonella typhimurium* has been confirmed. In contrast, autoxidized brassicasterol, 7-dehydrocholesterol, ergosterol, lanosterol, sitosterol and stigmasterol were non-mutagenic in the same assay, and 38 individual cholesterol derivatives, including many cholesterol autoxidation products, were also non-mutagenic. The mutagenic species from mutagenic cholesterol preparations were shown to be neutral steroids that are very much more polar than the cholesterol autoxidation products so far identified. High-performance liquid chromatography resolved mutagenic cholesterol preparations into several mutagenic and non-mutagenic fractions. The chemical nature of the mutagens is suggested as involving oxidation of the sterol B-ring and of the side-chain.

INTRODUCTION

We have demonstrated previously that naturally air-aged USP cholesterol becomes mutagenic towards test strains TA98, TA1537, and TA1538 of *Salmonella typhimurium* (the Ames test). The observed mutagenicity did not require prior metabolic activation but was demonstrated directly on methanol-solubles (chiefly cholesterol autoxidation products) recovered from autoxidized cholesterol samples by washing with methanol and concentrating the methanol washes. Furthermore, the same mutagenicity was observed with pure non-mutagenic cholesterol that had been oxidized by heating at 70°C for several weeks in air or by irradiation with ^{60}Co γ-radiation for several days (Smith, Smart & Ansari, 1979).

As the potential harm from sterol mutagens in ingested cholesterol-containing foods is of interest, we have searched for the chemical species responsible for the observed mutagenicities. We describe here our efforts to isolate and characterize these mutagens. A preliminary account of some of these results has already been given (Ansari, Walker & Smith, 1980).

EXPERIMENTAL

Materials. Solvents for TLC were of reagent quality, and were redistilled prior to use. Solvents for HPLC were from Burdick and Jackson Laboratories, Muskegon, MI. The air-aged cholesterol used was USP cholesterol that had been stored in its original fibre drum container at room temperature for about 10 yr. Commercially available samples of brassicasterol (24-methyl-(24*S*)-cholesta-5,22-dien-3β-ol), 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol), ergosterol (ergosta-5,7,22-trien-3β-ol), lanosterol (5α-lanosta-8,24-dien-3β-ol), sitosterol (stigmast-5-en-3β-ol), and stigmasterol (stigmasta-5,22-dien-3β-ol) had also been stored in their original containers at room temperature, for 0·5–25 yr. Other oxidized cholesterol derivatives tested were commercially available (specially purified) or were synthesized in our laboratory for the purpose. With the exceptions noted, all sterols were chromatographically homogeneous by TLC and/or HPLC.

Mutagenic sterols. Samples of autoxidized cholesterol (or ergosterol) slurried in 6 vols methanol were stirred for 2 hr and each filtrate was concentrated under vacuum. Precipitated sterol was filtered off, and the methanol mother liquors were further concentrated under vacuum to precipitate additional sterol. After filtration of crystalline sterol, the mother liquors were evaporated under vacuum to dryness, yielding material for further work. A typical preparation from 200 g air-aged USP cholesterol yielded 10·4 g yellow solids containing cholesterol autoxidation products and derived mutagens (a typical dose-response with strain TA98 being 60, 120, 190, 230 and 280 revertants/plate with levels of 0·1, 0·5, 1·0, 2·0 and 4·0 mg/plate, respectively).

Analogous material was obtained from other sterols by dissolving the sterol in chloroform (3·5 g/5 ml), diluting with 30 ml warm methanol and filtering off the precipitated sterol. Evaporation of filtrates under vacuum gave material for further work. Sterol recoveries were: brassicasterol (25 yr old), 760 mg/g; ergosterol, 35 mg/g; lanosterol, 90 mg/g; sitosterol, 480 mg/g; stigmasterol, 310 mg/g. 7-Dehydrocholesterol did not give precipitated solids but was assayed as the unfractionated sample.

Additionally, samples of volatile material from air-aged cholesterol were collected by means previously described (van Lier, Da Costa & Smith, 1975; van Lier & Smith, 1970). Also, fractionation of mutagenic sterol preparations into acidic and neutral materials was achieved by dissolving the sterols mixture in 350 ml diethyl ether and washing the ether solution with 5% NaOH (or 10% Na_2CO_3) until the aqueous extracts were neutral. The ether layer was dried over

Abbreviations: CI = Chemical-ionization (mass spectrometry); EI = electron-impact (mass spectrometry); HPLC = high-performance liquid chromatography; TLC = thin-layer chromatography; t_R = retention time.

anhydrous sodium sulphate and evaporated under vacuum to give the neutral fraction. The aqueous layers were combined, acidified with 10% HCl and extracted with diethyl ether; evaporation of the dried ether extract under vacuum gave the acidic fraction.

Physical data. Physical data obtained in support of the identity and purity of sterol samples included melting points taken on a Kofler block under a microscope, infrared absorption spectra taken with a Perkin-Elmer Corp. Model 337 infrared spectrophotometer equipped with beam condenser, ultraviolet light absorption spectra using a Cary Model 14 instrument, and EI and CI mass spectra using a Finnigan Corp. Model 3200 quadrupole mass spectrometer by techniques previously described (Lin, 1980; Lin & Smith, 1978 & 1979). Ammonia, 25% ammonia in methane, and methane were used as reagent gases in CI mass spectral studies.

Chromatography. Fractionation of air-aged sterol preparations was conducted by means previously described (Ansari & Smith, 1979) for HPLC. Preparative HPLC of 2-g samples was conducted using Waters Associates (Milford, MA) Prep LC/500 equipment and preparative μBondapak C_{18} (reverse phase) or μPorasil (adsorption) cartridges. Solvent flow was at 100 ml/min, and effluent monitoring was by differential refractive index. Semi-preparative 1·2 cm × 30 cm μBondapak C_{18} columns were also used at flow rates of 2·0–3·0 ml/min, with effluent monitoring by 212-nm light absorption using a Perkin-Elmer Corp. Model LC-55 variable wavelength spectrophotometric detector and differential refractive index measurements. Bulk sterol fractionations were also conducted on 3·5 cm × 30 cm columns with 100 g Silica Gel 60 Silanized (E. Merck GmbH, Darmstadt, FRG) irrigated by gravity flow, and by preparative TLC using 1 mm thick 20 cm × 20 cm chromatoplates of Silica Gel PF_{254} (E. Merck GmbH) irrigated by ascending solvent flow several times.

Analytical HPLC was conducted using either 3·9 mm × 30 cm μBondapak C_{18} columns and a flow rate of 1·0 ml/min or two 3·9 mm × 30 cm μPorasil columns in tandem with flow rates of 1·0–3·0 ml/min. Analytical TLC was conducted on 0·25 mm thick 20 cm × 20 cm chromatoplates of Silica Gel HF_{254} (E. Merck GmbH) irrigated in ascending fashion several times. Component detection involved (a) examination under 254-nm light for absorbing steroids, (b) spraying with *N,N*-dimethyl-*p*-phenylenediamine for sterol hydroperoxides and (c) spraying with 50% aqueous sulphuric acid and warming to detect all components (Smith & Hill, 1972; Smith, Matthews, Price *et al.* 1967). In a few cases, gas chromatography on 3% SE-30 and 3% OV-1 (both from Supelco Inc., Bellefonte, PA) was also used for component identifications (van Lier & Smith, 1968).

Chromatographic retention data are expressed as retention times (t_R) in minutes. Protocols developed for the fractionation of mutagenic materials are described in detail in the Results section.

Mutagenicity assay. Sterols were tested for mutagenicity against *S. typhimurium* test strains TA98, TA100, TA1535, TA1537 and TA1538 by the general methods of Ames (Ames, McCann & Yamasaki, 1975) as modified by us for sterols (Smith *et al.* 1979). In order to demonstrate dose-responses, samples were tested at levels of 0·1–5·0 and 5·0–40·0 mg/plate for individual pure sterols, 0·5–5·0 mg/plate (generally) for oxidized sterol mixtures, and 0·05, 0·1, 0·5 and 1·0 mg/plate for chromatography fractions. Tests were conducted in duplicate at 37°C for 48 hr and the results were averaged. Signs of toxicity were noted in a few cases, particularly at the higher test levels. Control levels of spontaneous revertants for the TA98 test strain used averaged 25 revertants/plate.

Positive mutagenicity was concluded only where the number of revertants exceeded the control spontaneous level by a factor of 2 and where dose-responses were evident over a suitable range of levels tested (Smith *et al.* 1979).

RESULTS

Mutagenic cholesterol preparations

Methanol-soluble material derived from naturally air-aged USP cholesterol was mutagenic towards the several *S. typhimurium* test strains, thus confirming our previous findings (Smith *et al.* 1979). The mutagenic sterol preparations were shown by chromatography to be very complex mixtures of cholesterol, recognized cholesterol autoxidation products and unidentified very polar sterol derivatives. TLC and HPLC patterns of the mutagenic samples revealed all the complexity of naturally air-aged cholesterol that we have previously described (Ansari & Smith, 1979; Smith *et al.* 1967).

Other sterols

In striking contrast to autoxidized cholesterol, methanol-soluble materials from air-aged brassicasterol, 7-dehydrocholesterol, ergosterol, lanosterol, sitosterol and stigmasterol were non-mutagenic in every test conducted. Moreover, mutagenicity was not observed at levels as high as 1·2 mg/plate in tests of volatile materials derived from air-aged cholesterol. Acidic material recovered from mutagenic batches of air-aged cholesterol was also found to be non-mutagenic.

Tests conducted on 38 pure cholesterol derivatives uniformly failed to reveal positive mutagenic responses at test levels as high as 40 mg/plate. The sterol derivatives tested included the epoxides cholesterol 5α,6α-epoxide (5,6α-epoxy-5α-cholestan-3β-ol) and cholesterol 5β,6β-epoxide (5,6β-epoxy-5β-cholestan-3β-ol), the steroid alcohols chol-5-en-3β-ol, chol-5-ene-3β,24-diol, cholest-5-ene-3β,4α-diol, cholest-5-ene-3β,4β-diol, cholest-5-ene-3β,7α-diol, cholest-5-ene-3β,7β-diol, (20*S*)-cholest-5-ene-3β,20-diol, cholest-5-ene-3β,25-diol, 5α-cholestane-3β,6β-diol, 5α-cholestane-3β,5,6β-triol, cholesta-4,6-dien-3β-ol, 5α-cholest-6-ene-3β,5-diol and 3α,5-cyclo-5α-cholestan-6β-ol, the sterol hydroperoxides 3β-hydroxycholest-5-ene-7α-hydroperoxide, 3β-hydroxycholest-5-ene-7β-hydroperoxide and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide, the steroid ketones cholest-5-en-3-one, cholest-4-en-3-one, cholesta-1,4-dien-3-one, cholesta-4,6-dien-3-one, cholesta-3,5-dien-7-one, 5α-cholestane-3,6-dione, cholest-4-ene-3,6-dione, pregn-4-ene-3,20-dione, 6β-hydroxycholest-4-ene-3-one, 3β-hydroxy-5α-cholestan-6-one, 3β,5-dihydroxy-5α-cholestan-6-one, 3β-hydroxycholest-5-en-7-one, 3β-hydroxypregn-5-en-20-one and 3β-hydroxy-27-

norcholest-5-en-25-one, the steroid hydrocarbons cholest-5-ene, cholesta-2,4-diene and cholesta-3,5-diene and the steroid acids 3β-hydroxychol-5-enic acid, 3β-hydroxy-22,23-bisnorchol-5-enic acid and 3β-hydroxyandrost-5-ene-17β-carboxylic acid. None of these steroids was mutagenic in our testing, but several were toxic towards the test bacteria, particularly cholest-5-ene-3β,25-diol, cholest-5-en-3-one, pregn-4-ene-3,20-dione, cholesta-2,4-diene and cholesta-3,5-diene.

In two instances commercially available steroid samples, tested as received, were found to be mutagenic. A 3β,5-dihydroxy-5α-cholestan-6-one preparation showed dose-response mutagenicity towards strain TA1538 (4·5 mg gave an 11-fold increase in revertants/plate). Likewise, a cholest-4-ene-3,6-dione sample was mutagenic towards strain TA98 (5·0 mg giving a threefold increase in revertants). However, highly purified samples of these mutagenic preparations, commercially available samples from other sources, and pure steroid 6-ketones synthesized in our laboratories were non-mutagenic in further testing. TLC and HPLC of the mutagenic preparations separated pure non-mutagenic steroid 6-ketones from the mutagenic components, but extensive attempts to recover the mutagenic components by chromatographic techniques were unsuccessful and the mutagens remain unidentified.

Fractionation of mutagenic cholesterol

Mutagenic preparations of autoxidized cholesterol were fractionated using various chromatographic processes and chemical separation into acidic and neutral materials. Several chromatographic processes yielded mutagenic material, but others resulted in a total loss of mutagenicity. Three of the most effective fractionation protocols utilized reverse phase chromatography in combination with separation into neutral and acidic materials.

Protocol I involved initial preparative HPLC on μBondapak C_{18} of 2·0 g batches of mutagenic cholesterol methanol-solubles, using methanol–water (9:1, v/v) for irrigation. The elution curve showed three discrete peaks that were collected separately. A fourth fraction consisted of a 100%-methanol wash of the column. Corresponding fractions from three identical chromatograms were pooled and evaporated under vacuum, yielding the following fractions: I-1, 2·935 g (48·9%), t_R 2–16 min, mutagenic (1·0 mg, 150 revertants/plate); I-2, 0·685 g (11·4%), t_R 16–25 min, weakly mutagenic; I-3, 0·523 g (8·7%), t_R 25–32 min, non-mutagenic; I-4, 1·589 g (26·5%), t_R 32–60 min, non-mutagenic. Mutagenic fraction I-1 (2·382 g) was separated into acidic (1·323 g, non-mutagenic) and neutral (1·101 g, mutagenic) materials, and 500 mg neutral material was rechromatographed semi-preparatively (20 mg at a time) on μBondapak C_{18} with methanol–water (1:1, v/v) at 3·0 ml/min, to give a complex elution curve of over 30 components (Fig. 1). Components were collected and combined arbitrarily for testing into ten fractions, as shown in Fig. 1, a terminal undiluted-methanol wash of the column constituting an eleventh fraction. Data for the 11 fractions were: I-1-1, 9 mg (1·8%), t_R 1·5–9·5 min, weakly mutagenic; I-1-2, 1 mg (0·2%), t_R 9·5–11·0 min, weakly mutagenic; I-1-3, 4 mg (0·8%), t_R 11·0–13·5 min, non-mutagenic; I-1-4, 3 mg (0·6%), t_R 13·5–15·5 min, non-mutagenic; I-1-5, 11 mg (2·2%), t_R 15·5–23·0 min, mutagenic (1·0 mg, 150 revertants/plate); I-1-6, 15 mg (11%), t_R 23·0–27·0 min, weakly mutagenic; I-1-7, 10 mg (2%), t_R 27·0–33·5 min, weakly mutagenic; I-1-8, 10 mg (2%), t_R 33·5–39·5 min, weakly mutagenic; I-1-9, 2·2 mg (0·4%), t_R 39·5–52·0 min, weakly mutagenic; I-1-10, 25 mg (5%), t_R 52·0–73·0 min, weakly mutagenic; I-1-11 (100%-methanol wash), 346 mg (67·2%), t_R 73 min, non-mutagenic).

Rechromatography of the weakly mutagenic second fraction I-2 (200 mg) semi-preparatively on μBondapak C_{18} with acetonitrile–water (9:1, v/v) at 2·5 ml/min gave four fractions, only the first of which (6 mg) was mutagenic. The three subsequent fractions (109 mg) were non-mutagenic.

Protocol II duplicated the first protocol except that acetonitrile–water mixtures were used. Initial preparative HPLC of 2·0 g mutagenic sterols on μBondapak C_{18} with acetonitrile–water (9:1, v/v) gave four fractions, as did Protocol I. The fractions were: II-1, 942 mg (47·1%), t_R 0–20 min, mutagenic (1·25 mg gave 225 revertants/plate and 2·50 mg, 350 revertants/plate); II-2, 216 mg (10·8%), t_R 20–30 min, non-mutagenic; II-3, 169 mg (8·5%), t_R 30–41 min, non-mutagenic; II-4, 306 mg (15·3%), t_R 41–70 min, non-mutagenic. Mutagenic fraction II-1 (900 mg) was separated into acidic (165 mg, non-mutagenic) and neutral (468 mg, mutagenic, 1·0 mg giving 280 revertants/plate) materials, and 464 mg neutral material was rechromatographed in several batches on analytical μBondapak C_{18} columns with acetonitrile–water (3:2, v/v) at 3·0 ml/min. A complex elution curve of over 30 components was obtained, and fractions were collected and combined arbitrarily into six fractions and tested, together with a 100%-acetonitrile column wash (fraction 7), as follows: II-1-1, 90 mg (19·4%), t_R 0–8·5 min, weakly mutagenic; II-1-2, 100 mg (21·5%), t_R 8·5–18·0 min, weakly mutagenic; II-1-3, 40 mg (8·6%), t_R 18·0–29·5 min, non-mutagenic; II-1-4, 20 mg (4·7%), t_R 29·5–33·5 min, non-mutagenic; II-1-5, 10 mg (2·6%), t_R 33·5–46·0 min, non-mutagenic; II-1-6, 100 mg (21·5%), t_R 46·0–55·0 min, non-mutagenic; II-1-7, 20 mg (4·3%), t_R 55·0–74·0 min, non-mutagenic.

Protocol III involved initial separation of 6·0 g mutagenic sterols into acidic (1·5 g, non-mutagenic) and neutral (4·235 g, mutagenic) materials. Chromatography of 2·0 g neutral materials on Silica Gel 60 Silanized gave fraction III-1 with 800 ml hexane–chloroform (9:1, v/v), 800 ml hexane–chloroform (4:1, v/v), and 800 ml hexane–chloroform (1:1, v/v), 12 mg, not tested; III-2 with 800 ml 100% chloroform, 807 mg (40·4%), non-mutagenic but toxic; III-3 with 400 ml ethyl acetate, 989 mg (49·5%), mutagenic; III-4 with 400 ml ethyl acetate and 800 ml acetone, 350 mg (17·5%), weakly mutagenic; III-5 with 800 ml methanol, 135 mg (6·8%), non-mutagenic. Mutagenic fraction III-3 (739 mg) was rechromatographed semi-preparatively on μBondapak C_{18} with methanol–water (1:1, v/v) at 2·0 ml/min, to give a complex elution curve of at least 30 components. Fractions were collected and arbitrarily combined for testing as follows: III-3-1, 15 mg (2%), t_R 4·0–14·0 min, non-mutagenic; III-3-2, 3·0 (0·4%), t_R 14·0–18·0 min, non-mutagenic; III-3-3, 10 mg (1%), t_R 18·0–44·0 min, non-

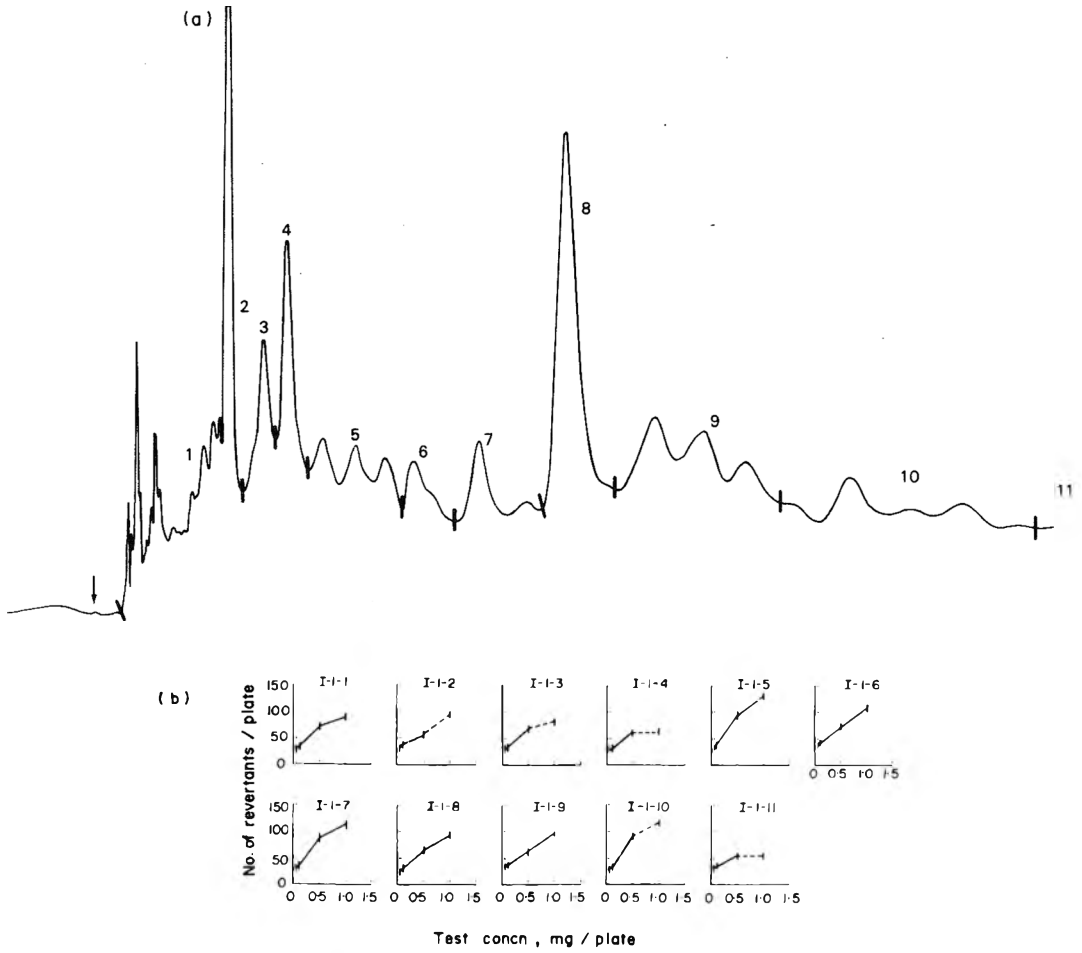

Fig. 1. (a) High-performance liquid chromatography of fraction I-1 neutral materials on μBondapak C_{18} with methanol–water (1:1, v/v) at 3·0 ml/min. (b) Results of mutagenicity assays in *Salmonella typhimurium* strain TA98 for the 11 fractions identified in (a). Data are averages of duplicate bioassays except for those for fractions I-1-2, I-1-3, I-1-4 and I-1-10 at the 1·0-mg/plate level (single assays). Toxicity was observed at 1·0 mg/plate with fraction I-1-11.

mutagenic; III-3-4, 8 mg (1·1%), t_R 44·0–51·0 min, non-mutagenic; III-3-5, 3·0 mg (0·4%), t_R 51·0–55·0 min, non-mutagenic; III-3-6, 11·0 mg (1·5%), t_R 55·0–62·0 min, non-mutagenic; III-3-7, 9·0 mg (1·2%), t_R 62·0–80·0 min, non-mutagenic; III-3-8, 17 mg (2·3%), t_R 80·0–98·0 min, non-mutagenic; III-3-9, 5·0 mg (0·7%), t_R 98·0–117 min, non-mutagenic; III-3-10 (100%-methanol wash), 235 mg (31·7%), t_R 117–146 min, mutagenic.

Rechromatography of the final fraction III-3-10 (230 mg in nine batches) on analytical μBondapak C_{18} columns with methanol–water (7:3, v/v) gave eight fractions and a methanol wash, as follows: III-3-10-1, 4 mg, non-mutagenic; III-3-10-2, 8 mg, toxic; III-3-10-3, 4 mg, mutagenic; III-3-10-4, 4 mg, mutagenic; III-3-10-5, 4 mg, weakly mutagenic; III-3-10-6, 8 mg, non-mutagenic; III-3-10-7, 10 mg, non-mutagenic; III-3-10-8, 11 mg, non-mutagenic; III-3-10-9, 6 mg, non-mutagenic. The three mutagenic middle fractions were individually rechromatographed in the same system, and the major components were characterized by CI mass spectra. Fractions III-3-10-3 and III-3-10-4 were characterized by essentially the same ions—*m/z* 482 (3·5%), 480 (9·0%), 478 (7·2%), 476 (9·1%), 465 (13·5%), 464 (18·9%), 463 (16·2%), 462 (16·2%), 447 (45·9%), 445 (63.0%), 431 (100%), 429 (73·8%), 415 (71·1%), 413 (57·6%), 399 (32·4%), 397 (36·0%), 375 (73·8%), 373 (84·6%), 359 (28·8%), 357 (28·8%), 345 (64·8%), 331 (81·0%), 316 (26·1%), 314 (15·3%), 312 (15·3%), 305 (18·0%), 303 (12·6%), 297 (13·5%), 289 (37·8%), 287 (23·4%), 273 (20·0%), 271 (21·8%) and 255 (16·2%).

Fraction III-3-10-5 resolved into two components upon final chromatography. The CI mass spectrum of the first component included the ions *m/z* 452 (55%), 450 (39%), 448 (55%), 433 (72%), 431 (72%), 416 (100%), 402 (50%), 400 (50%), 398 (39%) and 383 (28%), and that of the second the ions *m/z* 434 (16%), 432 (16%), 417 (100%), 416 (36%), 398 (28%), 383 (42%) and 368 (12%).

Rechromatography of several mutagenic fractions in different systems generally confirmed that mutagenic materials were present in the very polar fractions but no improvement on the three detailed descriptions for resolution of the mutagenic materials is yet at hand. Rechromatography of several non-mutagenic fractions did not yield mutagenic materials.

Thus each of these three protocols gave mutagenic materials, but none gave isolated homogeneous mutagenic steroid derivatives, and each at present has

serious limitations. Protocols I and II gave the same weight recoveries in initial preparative HPLC (47–49%) and in separation into neutral materials (46–52%), but in subsequent HPLC, losses were incurred in protocol I and only weakly mutagenic material was recovered in protocol II. Protocol III gave higher weight recoveries through separation into neutral material and reverse-phase chromatography, and subsequent HPLC gave more highly purified fractions retaining higher mutagenicities. On balance, protocol III appears the most effective.

Isolation of individual autoxidation products

Several oxidized sterol derivatives were recovered from more mobile non-mutagenic fractions of air-aged (autoxidized) mutagenic cholesterol preparations. Thus, the previously described fraction I-4 (300 mg) was subjected to preparative TLC with three ascending irrigations with benzene–ethyl acetate (3:2, v/v), and 41 mg sterols with the mobility of the cholesterol 5,6-epoxides was recovered. Rechromatography on μBondapak C_{18} with acetonitrile–water (9:1, v/v) at 1·0 ml/min gave a fraction with the same retention properties as authentic cholesterol 5α,6α-epoxide and a fraction with retention properties of the isomeric cholesterol 5β,6β-epoxide. Rechromatography of both fractions using the same conditions gave pure sterols identified by CI mass spectra as identical with the appropriate authentic sterol. Identities were further established by reductions with lithium aluminium hydride and chromatographic identification versus authentic sterols of the unique product stenediols thereby obtained. Thus, from cholesterol 5α,6α-epoxide was derived 5α-cholestane-3β,5-diol, and from cholesterol 5β,6β-epoxide were derived 5β-cholestane-3β,5-diol and 5α-cholestane-3β,6β-diol (Smith & Kulig, 1975). The HPLC elution curves showed a cholesterol 5α,6α-epoxide content of 83 μg/g air-aged cholesterol and a cholesterol 5β,6β-epoxide content of 75 μg/g.

After elution of the cholesterol 5,6-epoxides from μBondapak C_{18} with acetonitrile–water (9:1, v/v), continued elution yielded a sterol with the chromatographic properties (including a blue colour with 50% sulphuric acid spray) of cholest-5-ene-3β,4β-diol. Rechromatography on two μPorasil columns in tandem irrigated with hexane–isopropyl alcohol (24:1, v/v) at 2·0 ml/min gave pure cholest-5-ene-3β,4β-diol, m.p. 175–176°C, identified by chromatographic and EI and CI mass-spectrometric data in comparison with an authentic sample. The level of cholest-5-ene-3β,4β-diol found was 180 μg/g air-aged cholesterol.

A specific search for the epimeric cholest-5-ene-3β,4α-diol was also conducted using methanol-solubles from 44·0 g USP cholesterol processed in the same manner as that used for recovery of cholest-5-ene-3β,4β-diol. Material (15 mg) eluted from the region (R_F 0·29) occupied by cholest-5-ene-3β,4α-diol (were it present) on thin-layer chromatograms irrigated with benzene–ethyl acetate (3:1, v/v) (cholesterol R_f 0·69, cholest-5-ene-3β,4β-diol R_F 0·39) was chromatographed on two μPorasil columns in tandem using hexane–isopropyl alcohol (24:1, v/v) at 2.0 ml/min. Material eluting with the retention properties of cholest-5-ene-3β,4α-diol was then rechromatographed on μPorasil with hexane–isopropyl alcohol (49:1, v/v) at 3.0 ml/min. These systems provided base-line resolution of the epimers (cholest-5-ene-3β,4α-diol—t_R 25·8 min, cholest-5-ene-3β,4β-diol—t_R 22·0 min and cholesterol—t_R 6·0 min). Material (c. 500 μg) recovered with retention properties of cholest-5-ene-3β,4α-diol was rechromatographed by TLC using benzene–ethyl acetate (3:1, v/v), but no component could be detected in the region occupied by cholest-5-ene-3β,4α-diol (limit of detection estimated at *c.* 0·5 μg/g air-aged cholesterol).

It has not previously been recognized that commercially available samples of cholest-5-ene-3β,4β-diol routinely contain about 5% (w/w) of the epimeric cholest-5-ene-3β,4α-diol, as shown by chromatography on μPorasil with hexane–isopropyl alcohol (49:1, v/v), while cholest-5-ene-3β,4α-diol synthesized by lead tetraacetate oxidation of cholest-5-ene-3-one and subsequent reduction (Fieser & Stevenson, 1954) contains small amounts of cholest-5-ene-3β,4β-diol.

In another study, 1·0 g methanol-solubles were subjected to preparative HPLC on μPorasil irrigated with hexane–isopropyl alcohol (19:1, v/v). Six component peaks were detected, the second of which (t_R 8–12 min) was cholesterol. The third component (t_R 12–20 min) contained several oxidized cholesterol derivatives which were separated by preparative TLC using four ascending irrigations with benzene–ethyl acetate (3:1, v/v). From the most mobile zone was recovered a cholest-5-ene-3β,24-diol (probably cerebrosterol) and chol-5-en-3β-ol, and from the second more mobile zone was recovered crystalline 3β-hydroxypregn-5-en-20-one, identified by chromatographic and CI mass spectral properties in comparison with authentic sterols. Material (13 mg) from the third zone was rechromatographed on two analytical μPorasil columns in tandem with hexane–isopropyl alcohol (24:1, v/v) at 1·0 ml/min, and yielded a pure steroid with t_R 24·25 min, λ_{max} 237 nm (in ethanol), and CI mass spectra showing *m/z* 418 (100%) $(M + NH_4)^+$, 401 (97%) $(M + H)^+$ and 383 (11%) $(M - H_2O + H)^+$ with 25% ammonia in methane as the reagent gas, and *m/z* 441 (7%) $(M + 41)^+$, 429 (21%) $(M + 29)^+$, 401 (100%) $(M + H)^+$, 383 (21%) $(M - H_2O + H)^+$, 381 (14%) $(M - H_2O - H)^+$, 367 (7%) $(M - 33)^+$ with methane as the reagent gas. In these properties and in TLC and HPLC properties this steroid was identical with authentic 6β-hydroxycholest-4-en-3-one. It was estimated from elution-curve peak areas that the level of 6β-hydroxycholest-4-en-3-one in air-aged cholesterol was approximately 87 μg/g.

From the most polar TLC zone was recovered cholest-5-ene-3β,25-diol (m.p. 175–178°C), further identified by comparison of its CI mass-spectral and chromatographic data with those of an authentic sample.

A final isolation study utilized a 1-kg batch of mutagenic USP cholesterol from which 1·399 g volatiles were recovered for mutagenicity testing. The mutagens and polar autoxidation products were recovered by methanol extraction in the usual manner, dissolved in diethyl ether and extracted with 10% NaOH until the water extracts were alkaline. The aqueous layer was acidified with 10% HCl and extracted with ether, and the ether extracts were dried

over anhydrous sodium sulphate and evaporated under vacuum, thereby yielding 2·12 g yellow solids containing steroid acids. Chromatography of 500 mg steroid acids on a 1 cm × 45 cm column of 20 g Silica Gel 60 Silanized gave the following fractions: (1) elution with 100 ml methanol–water (1:1, v/v), 109 mg; (2) with 100 ml methanol–water (7:3, v/v), 82 mg; (3) with 100 ml methanol–water (4:1, v/v), 213 mg; (4) with 100 ml methanol–water (9:1, v/v), 41 mg; (5) with 100 ml methanol, 48 mg; (6) with 100 ml chloroform, 2 mg (99% material balance). The second fraction contained steroid acids and was subjected to preparative TLC using ethyl acetate–isooctane–acetic acid (40:20:1, by vol.) in three ascending irrigations. The C_{20}-, C_{22}- and C_{24}-steroid acids recovered unresolved from one another (as 12 mg oil) were rechromatographed on μBondapak C_{18} with methanol–pH 2·2 water (7:3, v/v; pH adjusted with phosphoric acid) at 1·0 ml/min. Steroid acids eluted at 6·0, 9·8 and 25·8 min were identified by their chromatographic properties as 3β-hydroxyandrost-5-ene-17β-carboxylic acid (2 ng/g air-aged cholesterol), 3β-hydroxy-22,23-bisnorchol-5-enic acid (219 ng/g) and 3β-hydroxychol-5-enic acid (14 ng/g), respectively. The C_{22}- acid was further identified by its CI mass spectrum *m/z* 364 (26%) $(M + NH_4)^+$, 347 (17%) $(M + H)^+$ and 329 (100%) $(M - H_2O + H)^+$ using 25% ammonia in methane as the reagent gas. This spectrum was identical with that of an authentic sample of 3β-hydroxy-22,23-bisnorchol-5-enic acid.

DISCUSSION

Data presented here confirm our previously reported results demonstrating that autoxidized cholesterol is mutagenic towards strains of *S. typhimurium*. They also establish that the observed mutagenicity cannot be associated with any of the recognized autoxidation products of cholesterol. Although over 80 such autoxidation products have now been identified, all (except the steroid acids described in this paper and C_2–C_6 volatiles) have chromatographic mobilities ranging between that of 5α-cholestane-3β,5,6β-triol (or 5α-cholestan-3β,5,6β,7α-tetraol) as the most polar and that of cholesta-2,4,6,-triene (and cholesta-3,5,7-triene) as the most nonpolar. As the mutagens described here are even more polar, we conclude that none of the cholesterol autoxidation products so far identified accounts for the observed mutagenicities. This conclusion is supported by test data on related sterol derivatives.

Moreover, neither the volatiles nor the C_{20}–C_{24} steroid acids formed during cholesterol autoxidation are mutagenic. Clearly, the mutagens formed during cholesterol autoxidation are new, previously uninvestigated cholesterol oxidation products deserving of our interest.

Only two recognized cholesterol autoxidation products appear to have been examined by others for mutagenicity towards *S. typhimurium*. Both 3β-hydroxychol-5-enic acid (Silverman & Andrews, 1977) and cholesterol 5α,6α-epoxide (Kadis, 1978; Kelsey & Pienta, 1979; Reddy & Watanabe, 1979) are nonmutagenic in these systems.

Cholesterol 5α,6α-epoxide has been reported to be toxic to *S. typhimurium* strains (Blackburn, Rashid & Thompson, 1979), to be carcinogenic in mice (Bischoff, 1957, 1963 & 1969; Bischoff & Bryson, 1977), to damage chromosomes and initiate DNA repair synthesis in cultured human fibroblasts (Parsons & Goss, 1978), to display transforming activity in cultured hamster embryo cells (Kelsey & Pienta, 1979) and to suppress *de novo* sterol biosynthesis in a variety of cultured mammalian cells by specific inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (Kandutsch & Chen, 1978; Peng, Tham, Taylor & Mikkelson, 1979). However, it is obvious from all the test results that cholesterol 5α,6α-epoxide is not mutagenic to *S. typhimurium*, and neither is the isomeric cholesterol 5β,6β-epoxide.

Mutagenic materials were recovered by each of the three fractionation procedures described, but no marked increase in mutagenicity per milligram of material tested was observed. These results may derive in part from poor physical recovery of mutagens in each step, but we also suspect that the mutagenic sterol derivatives are unstable, decomposing during fractionation. Despite failure to identify any specific oxidized cholesterol derivative as mutagenic, some remarks about the chemical nature of the mutagens may be made. Since mutagenicity is induced in pure non-mutagenic cholesterol by air oxidation (Smith *et al.* 1979) and since volatiles from autoxidized cholesterol were non-mutagenic, it follows that the mutagens are steroidal in nature. Chromatographic properties suggest that the mutagenic sterols are very polar and are probably more highly oxidized cholesterol derivatives than have previously been encountered. Preliminary CI mass spectra support this contention.

The spectra of fractions III-3-10-3 and III-3-10-4 are dominated by pairs of ions separated from one another by 2 amu: thus *m/z* 482 and 480, 465 and 463, 464 and 462, 447 and 445, 431 and 429, and so on, suggesting a mixture of at least two major components. The separations between the two sets of ions associated with one component (*m/z* 482, 465, 464, 447, 431) or the other (*m/z* 480, 463, 462, 445, 429) are exactly those anticipated for polyfunctional steroids where ammonium adduct $(M + NH_4)^+$, protonated molecular ion $(M + H)^+$, molecular ion $(M)^+$ or substitution ion $(M + NH_3 - OH)^+$, and dehydrated protonated molecular ion $(M - H_2O + H)^+$ or elimination ion $(M - OH)^+$ (Lin, 1980; Lin & Smith, 1978, 1979) are found. From these data a component formulated as $C_{27}H_{44}O_6$ (M = 464) and one formulated as $C_{27}H_{42}O_6$ (M = 462) are suggested. Fraction III-3-10-5 resolved into two components on final chromatography, the first of which gave a CI mass spectrum that included the ions *m/z* 450, 433, 415 and 397 *inter alia*, and may be interpreted as probably representing $(M + NH_4)^+$, $(M + H)^+$, $(M - H_2O + H)^+$, and $(M - 2H_2O + H)^+$, thus a component formulated as $C_{27}H_{44}O_4$ (M = 432). The CI mass spectrum of the second component displayed ions *m/z* 434, 417, 416, and 399 *inter alia*, interpreted as probably $(M + NH_4)^+$, $(M + H)^+$, $(M)^+$ or $(M + NH_3 - OH)^+$, and $(M - H_2O + H)^+$, thus representing an oxidized sterol $C_{27}H_{44}O_3$ (M = 416).

Other interpretations are also possible, but the data on balance support the formation of these components as more highly oxidized non-acidic choles-

terol derivatives, $C_{27}H_{42-44}O_{3-6}$. Oxidation in the B-ring about the Δ^5-double bond is surely the case, but oxidation in the side-chain is also likely to be involved. The lack of mutagenicity of sitosterol, the B-ring autoxidation chemistry of which is identical to that of cholesterol (Yanishlieva, Marinova & Schiller, 1978; Yanishlieva, Schiller & Marinova, 1978), further suggests that side-chain structure may be important for the observed mutagenicity of autoxidized cholesterol.

Nonetheless, although the observed highly oxidized cholesterol derivatives were major components of the mutagenic fractions analysed, we offer no evidence that the observed mutagenicities are associated with the oxidized sterols detected by mass spectrometry. Indeed, our experiences with mutagenic batches of 3β,5-dihydroxy-5α-cholestan-6-one and cholest-4-ene-3,6-dione described here demand that such an association is not implied until homogeneous, well characterized steroids have been isolated and shown to be mutagenic.

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AFLATOXIN DISTRIBUTION AND TOTAL MICROBIAL COUNTS IN AN EDIBLE OIL EXTRACTING PLANT. I. PRELIMINARY OBSERVATIONS

J. A. ABALAKA and J. A. ELEGBEDE
Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

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Abstract—Groundnut (peanut) kernels, groundnut and cotton-seed pellets and groundnut and cotton-seed oils (crude and refined) were screened for aflatoxins. The groundnut kernels and groundnut and cotton-seed pellets were additionally examined for total microbial counts as well as for certain types of micro-organism. All samples contained aflatoxins (B_1, B_2, G_1 and G_2) while a quantitative estimation of aflatoxin B_1 revealed that all samples contained this aflatoxin in varying amounts. The study identified in substantial numbers several types of micro-organisms that have been associated with industrial and health hazards.

INTRODUCTION

Aflatoxin contamination is a problem associated with commodities such as nuts and grains that are usually preserved by reduction of water activity (a_w; a measure of the available water in a food system, derived from the relative humidity of the atmosphere with which the food is in equilibrium). The contamination is more likely to occur where the prevailing temperatures are high. The moulds that produce aflatoxin, usually *Aspergillus flavus* and *A. parasiticus*, compete best with other microflora when the a_w is marginal for preservation (i.e. 0·84–0·86) and when the temperature is in the relatively high (25–45°C) range (FDA Bureau of Foods, 1973; Hunter, 1969).

Because of the widespread occurrence of aflatoxin in groundnuts and cotton seeds and the potential hazards associated with consumption of aflatoxin-contaminated human foods and animal feeds, a survey was carried out in a plant that processes groundnut and cotton seed into edible oil. The authors were attracted to this investigation because products from this process (especially the oil and the pellets) are consumed on a large scale (by man and animals, respectively) and to the best of their knowledge no investigation into this aspect of health-related products has been undertaken in Nigeria recently.

This paper reports the findings of a preliminary study, to be followed later by more detailed studies on several phases of the project. It should be noted that the company concerned remains anonymous throughout this report under an agreement permitting the investigators to have access to the "manufacturers' secrets".

EXPERIMENTAL

Test samples. Samples of groundnut kernels, groundnut and cotton-seed pellets and groundnut and cotton-seed oils (crude and refined) were supplied by a local oil-extracting plant. The groundnut kernels were of two categories; category A represented those derived from government supply and stored in the factory premises, while category B represented kernels derived from various miscellaneous sources and used for processing just as they came into the premises. During extraction processes the two categories of kernel were often mixed. Information on the sources of the cotton seeds was very scanty. It was subsequently discovered that during processing these three raw materials (groundnut kernels of both categories and cotton seeds) were mixed in various ratios, but the exact ratios were not disclosed to the investigators.

Sample preparation for aflatoxin detection and determination. Each of the categories of groundnut kernel, in 100-g batches and without removal of the membranous coats, was first pounded in a porcelain mortar and then blended in a high-speed Waring Blendor (Braun AG, Frankfurt/Main, FRG). Cotton-seed samples were blended directly in a Waring Blendor without any initial pounding. As in the case of the groundnut kernels, 100-g samples were used. For groundnut and cotton-seed pellets, thorough mortar-pounding of samples (50-g portions) was carried out prior to blending in a Waring Blendor. Subsequent procedures for the extraction and purification of aflatoxin in all samples followed the FDA Bureau of Foods official method (Association of Official Analytical Chemists, 1975) for peanuts, peanut products and oil-containing products, using methanol–water (55:45, v/v).

Detection and estimation of aflatoxin. Aflatoxins B_1, B_2, G_1 and G_2 were qualitatively identified by thin-layer chromatography (TLC), the R_F values being compared with those of B_1, B_2, G_1 and G_2 standards each containing 10 μg aflatoxin/ml. Aflatoxin B_1 was quantitated on a Velasco fluorotoxin meter (Neotec Instruments Inc., USA) against a standard 20-ppb microcolumn preparation in chloroform–methanol (96:4, v/v), as described in the Instruction Manual. Although there are several other types of aflatoxin, attention was deliberately paid to these four aflatoxins, for which there were available standards. Furthermore, only aflatoxin B_1 was determined quantitatively at this stage of the investigation.

Table 1. *Qualitative detection of aflatoxin in samples of groundnuts and groundnut and cotton-seed pellets and oils with quantitative estimation of the aflatoxin B_1 levels*

Sample	Aflatoxin				Level of aflatoxin B_1 (μg/kg)
	B_1	B_2	G_1	G_2	
Groundnut A* kernels	+	+	+	+	600
Groundnut B* kernels	+	+	−	−	450
Groundnut pellets	+	+	+	+	860
Cotton-seed pellets	+	+	±	±	480
Groundnut oil—crude	+	+	+	+	98
—refined	±	±	±	±	9
Cotton seed oil—crude	+	±	+	±	65
—refined	±	±	±	±	24

+ = Present − = Absent ± = Trace

*Groundnut A was derived from government source(s) and groundnut B from various uncontrolled sources.

Sample preparation for total microbial (aerobic) counts. Total microbial counts were carried out only on the groundnut kernels, cotton seeds and groundnut and cotton-seed pellets. For all microbial counts, a 100-g sample was added to 100 ml 1% peptone water (pH 6·8; Difco Laboratories, Detroit, MI, USA) and the mixture was equilibrated for 8 hr, with occasional vigorous shaking. Serial (1/10) dilutions of all original samples were carried out in 1% peptone water, allowing a 5-min interval between each dilution in the series. This time interval was aimed at avoiding possible cell injury to the microbial population as it passed from higher to lower concentrations in the serial dilution process. Samples (0·1 ml) were taken from the serial dilutions with the aid of sterile serological pipettes and inoculated in triplicate into plate count agar (PCA; Difco) plates using the pour-plate technique. Plates were swirled to allow homogeneous distribution of the microbial population, incubated at 25°C for 24 hr, moved to 30°C for 24 hr and finally transferred to 37°C for 24 hr. At the end of 72 hr, colonies growing on the plates were counted using a colony counter (A. Gallenkamp & Co. Ltd, London, UK). This shifting of plates to a different temperature after each 24-hr incubation period was to promote maximum microbial growth.

RESULTS

Detection of aflatoxins and quantitation of aflatoxin B_1

The result of the qualitative detection of the four aflatoxins is shown in Table 1. All four aflatoxins were detected in the category A groundnut kernels, while two (B_1 and B_2) were detected in the category B kernels. The groundnut pellets showed all four aflatoxins while the cotton-seed pellets were positive for aflatoxins B_1 and B_2 and showed only traces of G_1 and G_2. The crude groundnut oil contained all four aflatoxins while the crude cotton-seed oil contained aflatoxins B_1 and G_1 with only traces of B_2 and G_2. Both the refined groundnut and refined cottonseed oils showed traces of all four aflatoxins.

All the samples showed some aflatoxin B_1 (Table 1), the highest set of values being recorded for the pellets and the lowest for the refined products. A striking observation was that the category A groundnut kernels from a government-controlled source(s) contained much more aflatoxin B_1 (600 μg/kg) than did the category B groundnut kernels derived from several miscellaneous sources (450 μg/kg). It is also noteworthy that although the crude groundnut oil had a much higher aflatoxin B_1 content than crude cotton-seed oil (98·0 *v.* 65 μg/kg), a higher B_1 level was recorded in refined cotton-seed oil (24 μg/kg) than in refined groundnut oil (9·0 μg/kg).

Microbial (aerobic) counts

In contrast to the relative levels of aflatoxins, the microbial counts were higher in the groundnut kernels of category B than in those of category A (Table 2). The lowest micro-organism count was recorded in the cotton-seed pellets.

Because of their involvement in diseases or in spoilage, Bacilli, Salmonellae, *Escherichia coli*, moulds and Pseudomonas organisms were selected for separate counting. No attempt was made in this study to identify individual species of these organisms except in the case of *E. coli*. The groundnut kernels in category B showed the highest counts of Baccillus, Salmonella and Pseudomonas organisms and *E. coli*, while groundnut kernels in category A showed the highest mould count. There was a significant drop in counts for the pellets compared with those for either category of kernel.

DISCUSSION

Although all four aflatoxins were detected in both categories of groundnut kernel (Table 1), it has not been possible to establish the origin of the contamination, that is to determine whether it occurred at the farm or during storage. This must be established in subsequent studies to make meaningful recommendations possible. Several factors could be responsible for the finding of a higher level of aflatoxin B_1 in category A groundnut kernels, from government sources, than in kernels of category B, from various sources (Table 1). One possibility is that the groundnut kernels from government sources may have come from cheap sources rather than from actual government-controlled farms, when demand exceeded the farm production. It is also possible that while the

Table 2. *Total and differential microbial (aerobic) counts on samples of groundnuts and groundnut and cotton-seed pellets*

Sample	Microbial count (no./100 g sample)*					
	Total	Bacillus spp.†	Salmonella spp.†	*Escherichia coli*†	Moulds†‡	Pseudomonas spp.†
Groundnut A§	2×10^7	405	10	15	200	6
Groundnut B§	$3{\cdot}5 \times 10^8$	420	16	25	86	14
Groundnut pellets	5×10^3	18	2	4	9	2
Cotton-seed pellets	4×10^2	11	ND	3	20	ND

ND = None detected

*Counts are means of three counts on each of three plates.

†Counts were made without pre-enrichment.

‡Moulds were counted as no. of colony-forming units/100 g.

§Groundnut A was derived from government source(s) and groundnut B from various uncontrolled sources.

groundnuts may have been relatively free of contamination when delivered from government sources, storage under appropriate conditions of humidity and temperature may have caused rapid mould growth and the production of substantial levels of aflatoxin(s). Further, potent producers of aflatoxin B_1 may have been more numerous in groundnuts from government sources than in groundnuts from other sources, although this would probably be an unusual situation.

Pellets, both of groundnut and cotton seeds, showed a high level of aflatoxin B_1 when compared with the other components studied. Groundnut pellets showed a much higher level than either category of kernel. This is not surprising since pellets represent the concentrated form of the solid. The aflatoxin originally present in the groundnut and cotton seeds would be partitioned between the oil phase and the pellets made from the water-containing residue. Thus, after processing, a greater amount of the aflatoxin would be pressed into the pellets than would be found in the oil phase.

The presence of such a high level of aflatoxin B_1 in pellets should be viewed with considerable concern since several feed-processing companies in this country use the pellets as major raw materials for the formulation and production of various poultry feedstuffs. Moreover, it has been shown that aflatoxin B_1 is a cancer-causing agent and that when it is ingested by laying hens it is in part transmitted unchanged to the egg (Jacobson & Wiseman, 1974). Thus, human subjects stand a chance of consuming a high level of aflatoxin B_1 through egg consumption. It may be argued that the heating that takes place during feedstuffs manufacture would destroy aflatoxin B_1 or any other aflatoxin present in the pellets, but it has been shown that in aflatoxin-contaminated cereal used for making bread, the aflatoxin level fell to only 50% of the initial level after exposure to baking temperature (Jemali & Lafont, 1972).

The finding that aflatoxin B_1 levels as high as 9·0 μg/kg (in refined groundnut oil) and 24 μg/kg (in refined cotton-seed oil) were recorded in the ready-to-consume products should also cause concern. These oils are consumed almost on a daily basis in this part of the world and, therefore, they could have a cumulative effect on the organs of the body. Moreover, the incidence of hepatocarcinogenesis shows a trend in parallel with aflatoxin B_1 consumption (Shank, Gordon, Wogan *et al.* 1972).

The fact that the microbial counts were considerably lower in the pellets than in the kernels may be attributed to the difference between the water activity of the two sets of samples. However, the high microbial counts observed in the samples were not as significant as the types of micro-organism found (Table 2). The presence of substantial numbers of Salmonella, *E. coli*, Bacillus and Pseudomonas species, and moulds were of particular interest to the investigators. While these were not the only microbial organisms in the samples, they were chosen because of their involvement in certain diseases, especially in poultry and piglets, and in some cases in human subjects. Salmonella organisms have been implicated in salmonellosis of farm animals and birds (Edel, Guinee, VanScholhorst & Kampelmacher, 1967; Ghosh, 1972; Harvey, 1973; Lee, 1973; Public Health Laboratory Working Group, Skovgaard & Nielson, 1972; Sinell, 1973; Snoeyenbos, Carlson, McKie & Symser, 1967), while the involvement of animal feedstuffs and poultry in the transmission of salmonellosis to man, in what has become known as the 'Salmonella cycle' of infection has been documented (Edel, Guinee, VanScholhorst & Kampelmacher, 1973; Grau & Smith, 1971; Hobbs, 1971). *Escherichia coli* has been shown to cause diseases in piglets under natural and experimental conditions (Julseth, Felix, Burkholder & Deibel, 1969; Tiwary & Prassad, 1972; Williams & Newell, 1967). Some species of Bacillus, for example *B. anthracis*, are pathogenic, some such as *B. cereus* are food-poisoning micro-organisms, while others, such as *B. subtilis*, cause miscellaneous problems. Some pseudomonads are known to be pathogenic as well as being associated with general microbiological problems under certain conditions. The pellets and kernels studied may serve as reservoirs for transmitting these micro-organisms to animals and man.

This preliminary investigation has therefore served as a warning of the high risks involved in adopting an attitude of indifference towards carrying out surveillance on products from local companies, and should stimulate interest in a more detailed investigation into the general distribution of both aflatoxins and micro-organisms from the starting point (possibly the farms) to the final (refined) products. It also reveals the need for thorough heat treatment of the pellets during feed

formulation and production. Furthermore products of this nature, consumed either directly or indirectly, may contribute significantly to the increasing number of deaths of unknown cause now being recorded in this country.

Conclusion

Aflatoxins have been identified in the products of an edible-oil processing plant in quantities sufficient to cause major concern, especially to the food regulatory agencies. Such findings should stimulate more active research into similar potential sources of health hazard.

The high level of aflatoxin detected in this investigation appears to suggest either a poor method of storage or a lack of thorough screening at the buying stage, or possibly both. However, a better picture of the cause of such levels of aflatoxins and other potential health hazards should emerge after a thorough survey of the entire plant has been carried out.

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MODIFICATION BY BEET AND CABBAGE DIETS OF AFLATOXIN B_1-INDUCED RAT PLASMA α-FOETOPROTEIN ELEVATION, HEPATIC TUMORIGENESIS, AND MUTAGENICITY OF URINE

J. N. BOYD, J. G. BABISH and G. S. STOEWSAND
*Institute of Food Science, Cornell University, Geneva, NY 14456, USA**

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Abstract—Weanling male Fischer rats were fed a purified diet or diets containing 25% (w/w) freeze-dried ground beets or cabbage with or without 1 ppm aflatoxin B_1 (AFB_1) for 26 wk. In 3–7 wk the cabbage diet diminished, while the beet diet enhanced AFB_1-induced plasma α-foetoprotein (AFP) elevation. When the experiment was extended to 42 wk by maintaining the animals on the purified (basal) diet for a further 16 wk the rats that had consumed AFB_1 in the beet diet had 72 ± 14 tumours/liver (mean surface diameter of tumours, 6·13 ± 4·69 mm); animals that had been given AFB_1 in the control diet had 30 ± 16 tumours/liver (mean surface diameter, 4·36 ± 3·16 mm); rats that had been given AFB_1 in the cabbage diet had 13 ± 5 tumours/liver (mean surface diameter, 4·28 ± 2·89 mm). In the Salmonella/mammalian microsomal test, urine from rats fed AFB_1 with beets caused significantly ($P < 0.05$) more revertants in *Salmonella typhimurium* strain TA98 than did urine from rats fed AFB_1 with purified or cabbage diets. The beet- and cabbage-containing diets had no effect on the plasma AFP concentration, hepatic tumorigenesis, or the mutagenicity of urine in rats receiving no AFB_1. The evidence suggests that non-nutrient components of common vegetables may influence the response to chemical carcinogens, and that AFP determinations are useful in the rapid identification of dietary factors that modify carcinogenesis.

INTRODUCTION

In the past decade there has been increasing interest in the possibility that components of food might influence carcinogenesis. Nutritional deficiencies, excesses and imbalances can play an important role in the aetiology of neoplastic disease (Werther, 1980). There is also evidence that non-nutrient components of foods can influence carcinogenesis in experimental animals, whether these components are naturally occurring or added intentionally (Wattenberg, 1975 & 1979; Wattenberg, Loub, Lam & Speier, 1976). Vegetables of the cabbage family, Cruciferae, have been shown to inhibit AFB_1-induced hepatic oncogenesis in the rat (Stoewsand, Babish & Wimberly, 1978). Compounds occurring naturally in cruciferous vegetables have also been shown to inhibit mammary carinoma induced by dimethylbenzanthracene, presumably by acting as inducers of the microsomal monooxygenase system (Wattenberg & Loub, 1978).

AFP, an α-1-globulin synthesized by the liver and yolk sac, is a major plasma protein of the foetus, but is found only at very low levels in adult plasma (Gitlin, 1975). High concentrations in adults have been associated with primary liver cancer, and transient elevations have been observed during pregnancy, following partial hepatectomy, and during various stages of a number of liver diseases (Sell, Becker, Leffert & Watabe, 1976; Watanabe, Miyazaki & Taketa, 1976). Studies of hepatocarcinogenesis in experimental animals often include determination of AFP, since its elevation has been observed within a few weeks following administration of a number of chemical carcinogens (Becker & Sell, 1979a,b; Jalanko & Ruoslahti, 1979). AFB_1 has been associated with early AFP elevation in rats (Kroes, Sontag, Sell *et al.* 1975; Kroes, Williams & Weisburger, 1973). It has recently been shown that in Fischer rats a cauliflower-containing diet that inhibited AFB_1-induced hepatocarcinoma also inhibited early AFP elevation induced by the AFB_1 (Boyd, Sell & Stoewsand, 1979).

The Salmonella/mammalian microsome test for mutagenicity is a widely used *in vitro* test for potential carcinogenicity of chemicals (Ames, McCann & Yamasaki, 1975). It has been proposed that mutagenesis testing of body fluids of treated animals could be an indirect *in vivo* screening procedure for potential carcinogens (Food Safety Council, 1978). This protocol might also be useful in the investigation of factors that modify tumorigenesis. Increased or decreased mutagenesis of blood or urine might be indicative of enhancement or inhibition of neoplastic disease.

This study was undertaken to investigate the effect of two vegetables, one cruciferous and one non-cruciferous, on hepatic tumour development induced in rats by AFB_1, and to determine whether the mutagenesis testing of urine and determination of plasma AFP levels could be useful in the early prediction of potential modification of neoplasia.

*The addresses of the individual authors are as follows: J. N. Boyd and G. S. Stoewsand, Department of Food Science and Technology, New York State Agricultural Experiment Station, Geneva, NY 14456; J. G. Babish, Department of Preventive Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.

Abbreviations: AFB_1 = Aflatoxin B_1; AFP = α-foetoprotein

EXPERIMENTAL

Sixty weanling male F344 Fischer rats (Charles River, Wilmington, MA) were housed individually in raised wire cages at 23°C on a 12-hr light/dark cycle. They were given, *ad lib.*, water and either modified AIN-76 purified diet (Bieri, Stoewsand, Briggs *et al.* 1977) or diets containing 25% (w/w) freeze-dried ground cabbage (*Brassica oleracea* L) or table beets (*Beta vulgaris* L) with or without 1 ppm AFB_1 (Calbiochem-Behring Corp., La Jolla, CA). Casein and carbohydrates were adjusted in the vegetable diets to make all of the treatments isocaloric and isonitrogenous (Table 1). Body weight and feed consumption were measured weekly.

At 1–3 wk intervals, until wk 11, blood was obtained from four animals from each dietary treatment group by cardiac puncture with a heparinized syringe, following the induction of light anaesthesia with ether or CO_2. Plasma AFP was measured by sandwich-type radioimmunoassay (Boyd, Stoewsand, Misslbeck *et al.* 1981).

During wk 26 of treatment, urine was collected for 8 hr from four animals per treatment group. It was filtered through Whatman No. 1 paper, and stored at −40°C. Mutagenic activity was determined by the Salmonella/mammalian microsomal test (Ames *et al.* 1975), using duplicate samples treated with β-glucuronidase from *Helix pomatia* (Sigma Chemical Co., St. Louis, MO) and *Salmonella typhimurium* strain TA98. Ampicillin (12·5 μg/ml) was added to the top agar to prevent the growth of contaminating organisms from the non-sterile urine. In plates for which metabolic activation was used, the S-9 fraction of liver homogenate from Aroclor-treated rats was added to the top agar. Colonies were counted using an NBS Biotran II, model CIII Automatic Colony Counter (New Brunswick Scientific, Edison, NJ).

After the 26-wk treatment period all of the animals were maintained on the purified (basal) diet without AFB_1 for a further 16 wk. At the end of this period the animals were anaesthetized with CO_2, blood was obtained for AFP determination, and the animals were killed by decapitation. The livers were excised, weighed, and examined for the presence of tumours. Tumours greater than 1 mm in diameter on the surface of the liver were counted and measured with calipers.

The MINITAB computerized statistical program was used to examine differences among treatment means by analysis of variance (Ryan, Joiner & Ryan, 1980). Significant differences were determined by the procedure of Waller & Duncan (1969).

RESULTS

The results of weekly weight determinations appear in Fig. 1. Diet consumption and body weights were significantly depressed ($P < 0·05$) in animals consuming the cabbage-containing diets, either with or without AFB_1, and in the animals consuming AFB_1 compared to animals receiving the same diet without AFB_1.

The weekly dose of AFB_1 (mg/kg body weight) received by each animal was estimated from food consumption data. The mean doses for each treatment group of total AFB_1 consumed per animal after 1, 5, 12 and 26 wk are shown in Table 2. There were no significant differences between the different groups given aflatoxin in either weekly or total dose throughout the study.

No differences were observed in the number of revertants of *S. typhimurium* (TA98) induced by treatment with urine from rats fed the various diets without AFB_1. As shown in Table 3, in unactivated samples there were no differences in the number of revertants induced by urine from AFB_1-treated animals in the three dietary groups; however, metabolically activated urine from beet-fed rats induced a significant increase in revertants compared with that from rats fed the basal diet or cabbage-containing diets. There was no difference between the other two groups in the number of revertants induced by metabolically activated urine.

The results of plasma AFP determinations between 3 and 11 wk of treatment are presented in Fig. 2. All

Table 1. *Composition of basal and vegetable-containing rat diets*

Component	Percentage of component in		
	Basal diet	Cabbage diet	Beet diet
Casein	20·0	18·5	17·0
Corn starch	15·0	15·0	15·0
Sucrose	25·0	14·0	15·0
Glucose	25·0	13·5	15·0
Non-nutritive fibre	5·0	4·0	3·0
Mineral mix*	3·5	3·5	3·5
Corn oil	5·0	5·0	5·0
DL-Methionine	0·3	0·3	0·3
Vitamin mix†	1·0	1·0	1·0
Choline bitartrate	0·2	0·2	0·2
Santoquin‡	0·0125	0·0125	0·0125
Freeze-dried vegetable	0	25·0	25·0

*AIN-76 mixture.
†AIN-76 mixture. Vitamin K_1 was also added to the diet at a level of 2 mg/kg.
‡Antioxidant (Monsanto Chemical Co., St. Louis, MO).

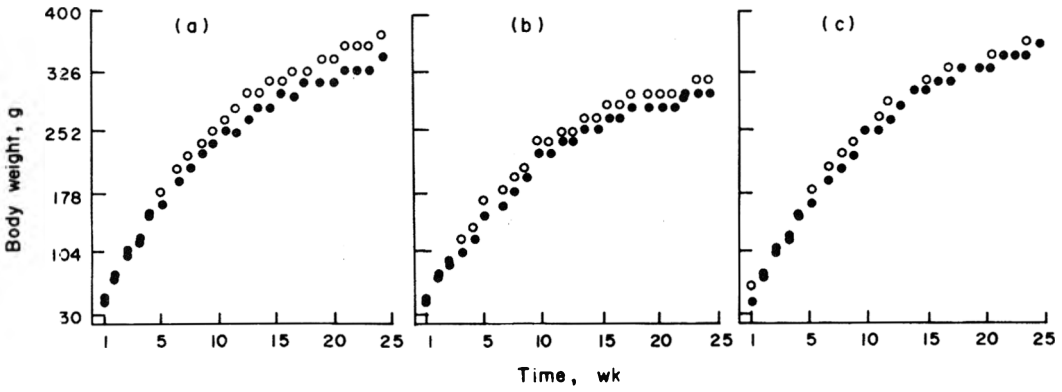

Fig. 1. Mean body weights of male Fischer rats fed (a) basal, (b) cabbage-containing or (c) beet-containing diets with (●) or without (○) 1 ppm aflatoxin B_1. (See Table 4 for no. in groups.)

diets without AFB_1 produced the normal lowering of the AFP level in the plasma with age of the rat. Between wk 5 and wk 11 animals consuming AFB_1 in the basal diet showed significantly higher AFP levels than all of the dietary groups not given AFB_1. Those consuming AFB_1 with cabbage had levels significantly higher than the groups not given AFB_1 only at wk 8, and had significantly lower plasma AFP levels than the other AFB_1-treated groups throughout the experiment. Animals consuming AFB_1 in the beet diet had significantly elevated AFP levels by wk 3, and the levels continued to be higher than those of the other two AFB_1-treated groups at all times except wk 5.

The survival rate (excluding early deaths), relative liver weights, and average number of tumours per liver are shown in Table 4. Two animals in the group given the beet diet plus AFB_1 died spontaneously, one during wk 36 and one during wk 40. Both had extensive liver tumours, and in one rat there was metastasis to lung tissue. Among the animals not given AFB_1, those consuming cabbage had significantly increased relative liver weight but no difference was observed between the beet- and basal-diet groups.

The measurements of the hepatic tumours are shown in Fig. 3. The mean, median and maximum size, and the number of tumours exceeding 10 mm in diameter were all significantly higher in the group fed the beet diet than in the other two dietary groups.

DISCUSSION

The apparent protective effect of the cabbage diet, as indicated by lowered AFP levels in blood, and hepatic tumorigenesis confirms earlier investigations that showed a similar protective effect of a cruciferous diet (Stoewsand *et al.* 1978). The increased relative liver weight in cabbage-fed animals indicated that these animals may have had an increased capacity to metabolize xenobiotic substances via enzyme induction (Gilbert & Golberg, 1965). Although AFB_1 must be metabolically activated to produce the ultimate carcinogen (Campbell & Hayes, 1976), increased metabolism does not necessarily lead to increased tumorigenesis, since different inducing conditions could lead to different pathways of metabolism or to a greater increase in excretion rate than in the synthesis of the ultimate carcinogen. The beet diet, without AFB_1, on the other hand, had no effect on relative liver weight. Preliminary investigations in this laboratory indicated that a 20% beet diet also had no effect on the activity of two hepatic microsomal monooxygenases, aminopyrine-*N*-demethylase or *p*-nitroanisole-*O*-demethylase. However, this does not preclude the possibility that dietary beets could alter the pattern of AFB_1 metabolism. The wide differences in relative liver weights observed in AFB_1-treated animals were indicative of differences in the amount of neoplastic tissue, rather than in the amount of normal hepatic tissue.

From Fig. 3 it can be seen that the maximum size and the number of hepatic tumours exceeding 10 mm

Table 2. *Estimates of the total doses of aflatoxin consumed by rats fed diets containing 1 ppm aflatoxin B_1*

Time (wk)	Dose/rat (mg/kg body weight) in rats fed		
	Basal diet	Cabbage diet	Beet diet
1	0·72 ± 0·03	0·67 ± 0·09	0·72 ± 0·10
5	3·11 ± 0·10	2·90 ± 0·28	3·02 ± 0·35
12	5·69 ± 0·23	5·60 ± 0·50	5·68 ± 0·59
26	9·48 ± 0·31	9·35 ± 0·59	9·43 ± 0·65

Values are means ± 1SD, estimated from the feed consumption of seven or eight animals per treatment.

Table 3. *Results of testing for mutagenicity, using the Salmonella/mammalian microsome test, 100-µl aliquots of urine from rats fed 1 ppm aflatoxin in basal, cabbage-containing or beet-containing diets*

Diet	No. of revertants/plate	
	−S-9	+S-9
Basal	14 ± 2	26 ± 7
Cabbage	14 ± 3	23 ± 11
Beet	14 ± 3	271 ± 99*

*Significantly different ($P < 0·001$) from other values in the same assay. Values are means ± 1 SD of duplicate assays of four urine specimens per dietary treatment.

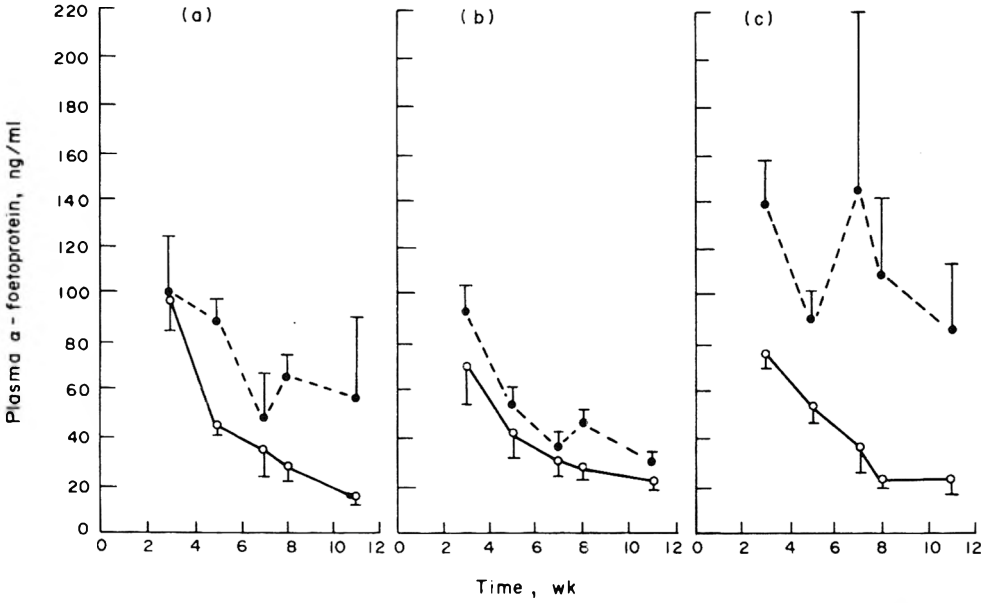

Fig. 2. Mean levels of α-foetoprotein in blood plasma in rats fed (a) basal, (b) cabbage-containing or (c) beet-containing diets with (●) or without (○) 1 ppm aflatoxin B_1.

were lower in the cabbage-fed group than in the group fed the basal diet, but the difference in mean tumour size between these two groups was not significant. More than 50 tumours had a diameter in excess of 10 mm, with a maximum 38·5 mm, in the beet-fed group. However, mean tumour size alone is not an adequate measure of the status of the animal, since the larger number of small tumours present in the animals in the groups fed beet or the basal diet, compared with those fed cabbage, accounted for part of the discrepancy between this parameter and others measured in this study. However, as shown in Table 4, there is a decrease in the mean number of tumours per liver in rats fed cabbage plus AFB_1, but an increased number of tumours per liver in the animals fed beet plus AFB_1. One rat in the group given the basal diet without AFB_1 died spontaneously during wk 19 and was found to have liver tumours. This is consistent with observations of an incidence of 1·7% of spontaneous liver tumours in male Fischer rats (Lijinsky, Reuber & Blackwell, 1980).

The results of mutagenesis testing indicate that following metabolic activation, there is an increase in the mutagenic capacity of the urine of rats fed beet plus AFB_1, while the cabbage diet did not affect the mutagenic response of urine (Table 3). These data indicate that the beet diet enhanced the production of AFB_1 metabolites that, in the presence of a metabolic activating system, were mutagenic in Fischer rats. Indeed, urinary mutagenesis testing correctly predicted the positive but not the negative dietary modification of hepatic tumorigenesis. The value of muta-

Table 4. *Survival, relative liver weights and numbers of tumours > 1-mm diameter on the surfaces of the livers of rats fed basal, cabbage-containing or beet-containing diets with or without 1 ppm aflatoxin B_1*

Diet	No. of rats surviving/total no. of rats	Relative liver weight (g/100 g body weight)	Mean no. of tumours
	Without aflatoxin B_1		
Basal	8/8	2·71 ± 0·08	0
Cabbage	8/8	3·13 ± 0·03*	0
Beet	9/9	2·72 ± 0·13	0
	With 1 ppm aflatoxin B_1		
Basal	11/11	4·71 ± 0·73	30 ± 16·
Cabbage	9/9	4·83 ± 0·69	13 ± 5*
Beet	6/8	8·26 ± 1·5*	72 ± 14*

Within aflatoxin treatment groups, values marked with an asterisk differ significantly (analysis of variance) from unmarked values ($P < 0·05$). The number of tumours in rats fed beet plus aflatoxin was significantly different ($P < 0·05$) from that in rats fed cabbage plus aflatoxin.

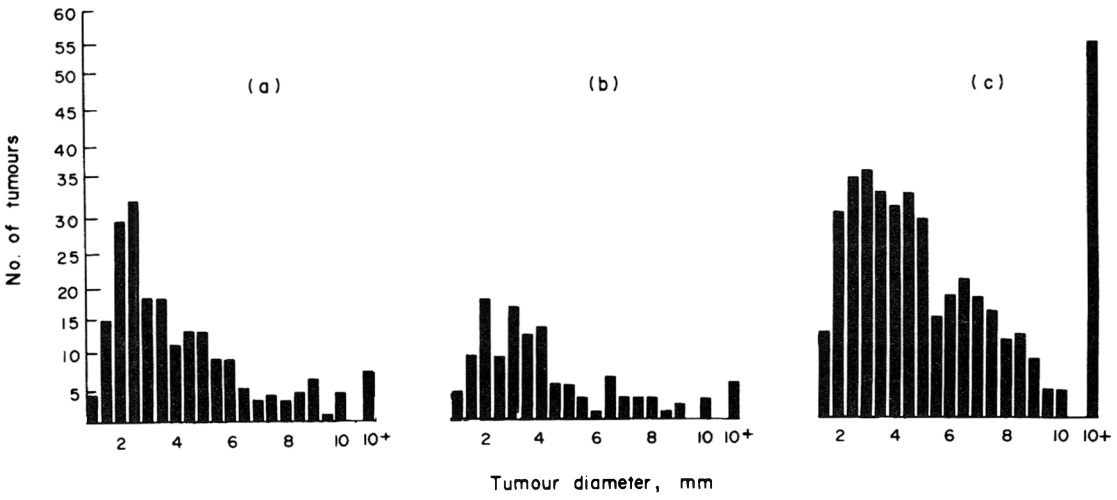

Fig. 3. Frequency distribution of different-sized hepatic tumours in rats fed (a) basal (n = 11), (b) cabbage-containing (n = 9) or (c) beet-containing (n = 6) diets. (a) Mean = 4·36 ± 3·16, median = 3·40, maximum = 25·65. (b) Mean = 4·28 ± 2·89, median = 3·36, maximum = 17·32. (c) Mean = 6·13 ± 4·69, median = 4·66, maximum = 38·55.

genesis testing of urine could, perhaps, be improved by collecting specimens on a weekly or bi-weekly basis during a short-term investigation.

In the AFB_1-treated animals, the cabbage diet resulted in decreased food intake, and thus decreased intake of AFB_1, but the beet diet resulted in no change in intake compared to the basal group. The difference in dietary intake, however, did not result in a significant difference between the dietary groups in the dose of AFB_1 (Table 2). Other investigators have demonstrated negative modification of tumorigenesis as a consequence of caloric restriction alone (Werther, 1980). Inadvertent caloric restriction could have contributed to the protective effect of the cabbage diet, but caloric intake cannot explain positive modification by the beet diet.

The crude fibre content of all of the diets was the same, but the concentration of individual fibre components varies in different plant products (Haard, 1976). These differences could cause differences in the absorption and faecal excretion of AFB_1 and its metabolites, as a consequence of increased faecal volume, decreased transit time through the gastrointestinal tract, or adsorption onto fibre components. In the present investigation, subjective observation indicated increases in faecal volume in both the cabbage- and beet-fed groups, compared to rats fed the basal diet. However, it is obvious that vegetable fibre, *per se*, is not a major contributor to the effect on tumorigenesis, since the beet and cabbage diets exhibited opposite effects on hepatic neoplasia. Since no tumours were found in rats consuming the beet diet without AFB_1, it can be concluded that beets do not contain components that initiate tumorigenesis, but that they may contain substances that promote the growth of AFB_1-induced neoplasms. Other non-initiating promoters of hepatic tumorigenesis have been described (Watanabe & Williams, 1978).

When the study was terminated, plasma AFP levels were less than 10 ng/ml for all animals that were not treated with AFB_1. Levels for animals consuming AFB_1 in the basal and cabbage diets exceeded 100 ng/ml, but were over 1000 ng/ml for animals in the group fed the beet diet. Actual values are not reported because the assay was developed for sensitivity in the low concentration range, and was not valid above 1000 ng/ml.

The differences in blood AFP levels are important for two reasons. Firstly the early differences in AFP concentration indicate that cabbage and beet diets exhibited their influence on tumorigenesis early in the process. Secondly, this investigation demonstrates that AFP determinations can be useful in the rapid identification of dietary factors that modify tumorigenesis. In the present investigation, differences in AFP concentration correctly predicted within a few weeks the differences in tumorigenesis observed in our long-term bioassay (Fig. 3).

The results of these experiments demonstrate that dietary cabbage acts as a 'negative modifier', and dietary beets act as a 'positive modifier' of AFB_1-induced hepatic tumorigenesis in male Fischer rats, and that plasma AFP and urinary mutagenesis determinations can be useful in the rapid identification of dietary factors that modify chemical carcinogenesis.

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THE COMPARATIVE METABOLISM AND TOXIC POTENCY OF AFLATOXIN B_1 AND AFLATOXIN M_1 IN PRIMARY CULTURES OF ADULT-RAT HEPATOCYTES

C. E. GREEN*, D. W. RICE, D. P. H. HSIEH and J. L. BYARD†

Department of Environmental Toxicology, University of California, Davis, CA 95616, USA

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Abstract—Both aflatoxin B_1 (AFB_1) and a hydroxylated metabolite, aflatoxin M_1 (AFM_1), were potent cytotoxins and genotoxins to primary cultures of rat hepatocytes. However, AFB_1 stimulated the release of lactate dehydrogenase into the culture medium and the loss of viable cells from the monolayer at lower doses than did AFM_1. The lowest toxic doses of AFB_1 and AFM_1 were 0·05–0·1 and 0·6 μg/culture, respectively. Genotoxicity, determined by an assay for stimulation of DNA repair, was apparent at lower doses than was cytotoxicity. AFB_1 was again more potent than AFM_1, stimulating DNA repair at 0·025 μg/culture, compared to the lowest genotoxic dose of AFM_1 of 0·05 μg/culture. At higher doses (1·2–2·4 μg/culture) the responses due to both aflatoxins in the cytotoxicity and DNA-repair assays were approximately equal. The metabolism of a low dose (c. 0·17 μg/culture) of [^{14}C]AFB_1 and [^{3}H]AFM_1 by cultured hepatocytes differed significantly. After 1 hr, 50% of the [^{14}C]AFB_1 remained unchanged in the culture medium, whereas about 18 hr were required for the same amount of [^{3}H]AFM_1 metabolism to occur. [^{14}C]AFB_1 was metabolized to AFM_1, to polar metabolites recovered in the aqueous phase after chloroform extraction, and to metabolites covalently bound to hepatocyte macromolecules. [^{3}H]AFM_1 was also metabolized to polar metabolites and to forms bound to macromolecules. The degree of covalent binding of the aflatoxins correlated with their cytotoxicity and genotoxicity at lower doses. After a 24-hr incubation, 12·5% of the dose of [^{14}C]AFB_1 was covalently bound to macromolecules compared to 1·5% of [^{3}H]AFM_1. Although AFM_1 was less potent than AFB_1 in cytotoxicity, DNA-repair and covalent-binding assays using primary cultures of hepatocytes, AFM_1 was still active at relatively low doses and therefore is probably a potent hepatotoxin *in vivo*.

INTRODUCTION

The hepatotoxicity and carcinogenicity of the mycotoxin, AFB_1, has been thoroughly documented (Campbell & Hayes, 1976; Wogan, 1973). However, relatively little is known about the toxic potency of the mammalian metabolites of AFB_1, such as AFM_1, a major hydroxylated metabolite which is invariably produced by animals exposed to AFB_1, including humans (Campbell, Caedo, Bulatao-Jayme, Salamat & Engel, 1970; Patterson, 1973). AFM_1 was the first aflatoxin metabolite to be identified (Allcroft & Carnaghan, 1963) and has been found in many human foods, such as milk from cows fed AFB_1-contaminated feed (Purchase, 1972). Therefore exposure to AFM_1 can occur in two ways, either as a metabolite formed after AFB_1 exposure or directly through the ingestion of food products from animals that have metabolized AFB_1 to AFM_1.

Few studies have been reported on the comparative toxicities of AFM_1 and AFB_1, although the data that have been collected suggest that AFM_1 is one of the most toxic of the AFB_1 metabolites. The two mycotoxins produced similar LD_{50} values and indistinguishable liver lesions during acute toxicity assays in 1-day-old ducklings (Purchase, 1967) and in rats (Pong & Wogan, 1971). Carcinogenicity studies of AFM_1 have been limited by the scarcity of the compound, but have been performed using rainbow trout and rats, two species that are very sensitive to AFB_1-induced neoplasia. Sinnhuber, Lee, Wales, Landers & Keyl (1974) found that AFM_1 was a potent hepatocarcinogen in rainbow trout, being about one third as carcinogenic as AFB_1. Synthetic AFM_1, on the other hand, had a considerably lower carcinogenic potency to rats than did natural AFB_1 (Wogan & Paglialunga, 1974).

The recent development of short-term bioassays for potential carcinogens has made it possible to estimate the toxic potency of chemicals using small quantities of purified compound. Most of these assays are based on the observation that the majority of carcinogens or a toxic metabolite of the carcinogen are genotoxic; that is, they bind covalently to DNA (Miller & Miller, 1974) and are presumed to produce a stable heritable change in the cell. Wong & Hsieh (1976) studied the potency of several aflatoxin metabolites in the most frequently used *in vitro* assay, the Ames Salmonella/mammalian microsome test and found that all aflatoxin metabolites tested were less mutagenic than AFB_1 itself. However, AFM_1 was the second most potent mammalian metabolite with 3·2% of the mutagenic activity of AFB_1.

Primary cultures of hepatocytes are another *in vitro* system that has been used to investigate the metabolism and toxic effects of carcinogens and cytotoxins.

*Present address: SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA.
†To whom requests for reprints should be addressed.

Abbreviations: AFB_1 = Aflatoxin B_1; AFM_1 = aflatoxin M_1; EDTA = ethylenediaminetetraacetic acid, tetrasodium salt; HPLC = high-pressure liquid chromatography; LDH = lactic dehydrogenase; TCA = trichloroacetic acid; TLC = thin-layer chromatography.

A carcinogen screening assay using these cells is based on the ability of the hepatocytes to metabolize chemicals to forms that react with DNA and then respond to the damage by repairing the DNA (Williams, 1977). With few exceptions, there is a good correlation between the carcinogenicity of a chemical and a positive response in the DNA-repair assay (Sirica & Pitot, 1980). Of all carcinogens, AFB_1 and its effects on cultured hepatocytes, the target cell *in vivo*, have been particularly well studied. Primary rat and mouse hepatocyte cultures have been shown to be a good model of *in vivo* metabolism and covalent binding, reflecting the difference in susceptibility of the two species to AFB_1 toxicity (Decad, Dougherty, Hsieh & Byard, 1979; Decad, Hsieh & Byard, 1977). These hepatocytes also respond to the cytotoxic effects of AFB_1, measured as the leakage of lactate dehydrogenase into the extracellular medium (Salcocks, Hsieh & Byard, 1981). Finally, DNA repair is stimulated in the hepatocytes by AFB_1 at relatively low concentrations (Michalopoulos, Sattler, O'Connor & Pitot, 1978; Williams, 1976 & 1977). Therefore, primary rat hepatocyte cultures were chosen as an appropriate model for these studies on the relative toxicity of AFM_1 to AFB_1. Using cultured hepatocytes, only small quantities of the purified test compound were required to carry out experiments on several aspects of aflatoxin toxicity—cytotoxicity, genotoxicity and metabolic fate.

EXPERIMENTAL

Animals. Male Sprague–Dawley rats, weighing 300–450 g at the time of the experiments, were purchased from Simonsen Laboratories (Gilroy, CA). The animals were fed Purina Laboratory Chow No. 5001 (Ralston Purina Co., Inc., St. Louis, MO) and water *ad lib.*

Isolation and culture of hepatocytes. Hepatocytes were isolated using a circulating perfusion system based on the method of Seglen (1976) and modified by Dougherty, Spilman, Green, Steward & Byard (1980). The cells were cultured in supplemented Waymouth's 752/1, a chemically defined culture medium designed to maintain cytochrome *P*-450 levels (Decad *et al.* 1977). The hormonal supplements in this medium also maintained DNA-repair enzyme levels (Sirica, Hwang, Sattler & Pitot, 1980). Modifications in the preparation of the perfusion buffers and the culture medium have been described previously (Green, Segall & Byard, 1981). Hepatocytes were cultured at a density of $2{\cdot}5 \times 10^6$ cells/4 ml culture medium/60-mm diameter dish. After 20–22 hr each culture contained $23{\cdot}9 \pm 1{\cdot}9$ μg DNA.

Chemicals. Unlabelled AFB_1 and AFM_1 were produced by cultures of *Aspergillus flavus* 3251 and purified according to the method of Stubblefield, Shannon & Shotwell (1970). The aflatoxins were free of ultraviolet-absorbing and fluorescent impurities as shown by TLC and HPLC.

Ring-labelled [^{14}C]AFB_1 was prepared from cultures of *Aspergillus parasiticus* ATCC 15517 supplemented with [^{14}C]acetate (New England Nuclear, Boston, MA) according to the procedure of Hsieh & Mateles (1971). The radiochemical purity was greater than 98% as determined by TLC and HPLC. Radiolabelled AFM_1 was prepared by biotransformation of [^{3}H]AFB_1 (Moravek Biochemicals, City of Industry, CA) using liver S-10 derived from male golden Syrian hamsters (Simonsen Laboratories). The preparation of the S-10 fraction has been previously reported (Hsieh, Wong, Wong *et al.* 1977). The radiochemical purity of the resulting [^{3}H]AFM_1 was greater than 96% based on TLC and HPLC.

The aflatoxins were added to cultures in propylene glycol. The maximum amount of propylene glycol used for any of the experiments was 0·3% of the total volume. [^{3}H]Thymidine was obtained from ICN (Irvine, CA) and had a specific activity of 72 Ci/mmol.

Cytotoxicity assay. Cytotoxicity was determined by measuring the leakage of the cytoplasmic enzyme LDH into the culture medium (Acosta, Anuforo & Smith, 1980) following a 24-hr exposure to AFB_1 or AFM_1. LDH was assayed in 50 μl of culture medium using the method of Wroblewski & LaDue (1955). Cytotoxicity was also demonstrated by counting the number of cells remaining attached to the dish following the chemical exposure period. Cells were counted at a magnification of × 200 in five 0·0025-mm^2 reticle fields, using the same fixed and stained cultures prepared for the autoradiography studies.

DNA-repair assay—liquid scintillation technique. The aflatoxins and 0·5 μCi/ml [^{3}H]thymidine were added to hepatocytes 20–22 hr after the initiation of the cultures. Control cells were exposed only to culture medium containing [^{3}H]thymidine and propylene glycol. Following a 24-hr incubation period, the culture medium was aspirated and the cells rinsed twice with 0·85% NaCl-1 mM thymidine and once with phosphate-buffered saline (Dulbecco & Vogt, 1954). The cultures were then frozen for later analysis.

To lower the background level of [^{3}H]thymidine incorporation into protein, the thawed cultures were treated with 2 ml of 0·05% trypsin (Sigma Chemical Co., St. Louis, MO) in 0·1 M-phosphate–0.1 mM-EDTA buffer for 30 min at 37°C. The cells and trypsin buffer were then removed from the dish and placed on ice. To recover essentially all of the cell material, the dish was rinsed with 1 ml phosphate-buffered saline which was added to the rest of the sample. Two aliquots were taken from each sample, one for DNA analysis and the other for radioactivity determination. Both aliquots were spiked with 500 μg bovine serum albumin precipitated with TCA, 10% final concentration, and chilled on ice for 30 min. The precipitate was collected on a Whatman GF/C glass-fibre filter (Whatman Ltd. Springfield Mill, England). The filter was washed thoroughly with 45 ml ice-cold 10% TCA by vacuum filtration to remove unincorporated [^{3}H]thymidine. For scintillation counting, the filter was heated with 0·2 ml NCS (Amersham, Arlington Heights, IL) at 50°C for 2 hr, cooled and vortexed with 10 ml Dimilume (Packard Instrument Co., Downers Grove, IL). The DNA content of each sample was determined in a duplicate filter by the method of Burton (1956) as modified by Richards (1974).

DNA-repair assay—autoradiography technique. The cultures were treated with AFB_1 or AFM_1 and [^{3}H]thymidine as described for the liquid scintillation technique. After carcinogen exposure, the cultures

were washed to remove unincorporated [^{3}H]thymidine as described above. The cells were then fixed for 1 min with methanol and stained with 2% acetoorcein (GIBCO, Grand Island, NY) for 10 min. Excess stain was removed by rinsing with distilled water. The dishes were air-dried and coated with NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY), diluted 1:1 (v/v) with distilled water. After a 7–10 day exposure period at 4°C in a desiccated, light-tight box, autoradiographs were processed with Kodak D19 developer for 4 min and with Kodak fixer for 5 min. Each dish was divided into thirds and 50 nuclei in each section were scored. The net grain/nucleus count was determined by subtracting the number of grains in a nuclear sized area of the cytoplasm of the same cell from the number in the nucleus. Any nucleus with more than six grains was considered to be exhibiting DNA repair, according to the criteria outlined by Brusick (1980) for determining a positive response in the DNA-repair–primary hepatocyte culture assay. Student's *t* test was used to determine which doses of AFB_1 and AFM_1 significantly ($P < 0.05$) elevated the response above the control level in the cytotoxicity and DNA-repair assays.

Metabolism and covalent binding studies. Approximately equivalent doses of [^{3}H]AFM_1 (0·168 μg/culture) and of [^{14}C]AFB_1 (0·170 μg/culture) were added to hepatocytes (isolated from the same rat) which had been in culture 20–22 hr. To determine the amount of non-enzymatic breakdown of the chemicals and of nonspecific binding, control cells were killed with 10% TCA before the addition of [^{3}H]AFM_1 or [^{14}C]AFB_1. Excess culture medium which was not added to cells was used as a zero-time sample. For metabolism studies, the culture medium was removed from triplicate plates for each mycotoxin after 1, 3, 6, 12 and 24 hr of incubation. Since one set of three control dishes was used for the entire time course, 0·2 ml of culture medium was removed from each TCA-killed control at 1, 3, 6 and 12 hr. The remaining medium was aspirated at 24 hr. All of the cultures were washed with 2 ml of ice-cold phosphate-buffered saline which was then pooled with the culture medium. The culture medium and washes were extracted with an equal volume of chloroform three times. The radioactivity in the chloroform phase and in the aqueous phase was determined at each time point for control cells and live cells, as well as zero-time samples. Because a significant amount of tritium exchange occurs during the 24-hr incubation with [^{3}H]AFM_1, the radioactivity recovered in the aqueous phase of live cells treated with either mycotoxin was corrected by subtracting the values obtained by incubating with TCA-killed hepatocytes.

Another aliquot of culture medium was extracted in the same way, and the quantity of unmetabolized aflatoxin determined in the chloroform extracts. The extracts were dried under nitrogen. AFM_1 samples were dissolved in benzene–acetonitrile (9:1, v/v) and AFB_1 samples were dissolved in benzene–acetonitrile (98:2, v/v).

Duplicate aliquots of these extracts were spotted on Whatman LK5 pre-absorbent TLC plates (Whatman Inc., Clifton, NJ) along with authentic standards of AFB_1 and AFM_1. The plates were developed in chloroform–acetone–isopropanol (85:15:2·5, by vol). Unmetabolized parent compound was quantitated by fluorescence spectrodensitometry using a Schoeffel SD300 densitometer (Schoeffel Instruments, Westwood, NJ).

Covalent binding was determined by measuring the radioactivity associated with TCA-precipitated macromolecules. The cells were scraped from the dishes in ice-cold phosphate-buffered saline and homogenized by repeated pipetting. Aliquots were withdrawn for DNA analysis (Burton, 1956; Richards, 1974). The remainder of the sample was precipitated with 10% TCA. The precipitated macromolecules were collected on GF/C glass-fibre filters (Whatman Ltd) and washed with ice-cold solvents—5 ml 10% TCA, 25 ml ice-cold methanol, and 15 ml ether—to remove loosely associated aflatoxin. The washes were collected to determine the recovery of radioactivity. The filters were digested with NCS (Amersham) and mixed with Dimilume scintillation fluid (Packard Instrument Co.). The radioactivity associated with TCA-killed cells which were incubated with radiolabelled aflatoxins for 24 hr was subtracted from all values for live cells. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer. [^{14}C]-Toluene and [^{3}H]toluene (Packard Instrument Co.) were used as internal standards for quench correction.

RESULTS

Cytotoxicity of AFB_1 and AFM_1

Both AFB_1 and AFM_1 were potent cytotoxins to cultured rat hepatocytes as indicated by increased LDH activity in the medium (Fig. 1) and by loss of cells from the monolayer with higher doses (Table 1). The lowest dose of AFB_1 that caused a significant ($P < 0.01$) increase in extracellular LDH activity was 0·05 μg/culture; 0·1 μg/culture was the lowest dose that resulted in a significant ($P < 0.005$) decrease in cell number. Significant toxicity due to AFM_1 was apparent at 0·6 μg/culture ($P < 0.0005$ for LDH release and $P < 0.005$ for cell counts in comparison with the

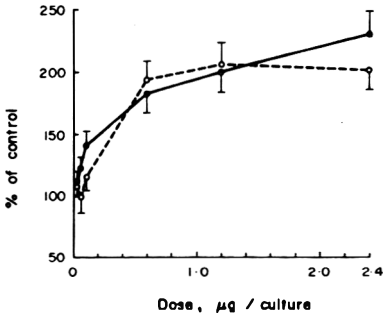

Fig. 1. Effect of aflatoxin B_1 (●——●) and aflatoxin M_1 (○– –○) on the leakage of lactate dehydrogenase into the culture medium of rat hepatocytes. Aflatoxin B_1 or aflatoxin M_1 were incubated with primary hepatocyte cultures for 24 hr before assaying the culture medium for lactate dehydrogenase. Cells were plated at a density of $2·5 \times 10^6$ viable cells/60-mm diameter dish/4 ml culture medium. Extracellular lactate dehydrogenase activity for control cells was 228 ± 32 units/ml (mean ± 1 SD). Values in the figure are means from quadruplicate cultures, and the range bars indicate the SEM.

Table 1. *Cytotoxicity of aflatoxin B_1 and aflatoxin M_1 in primary cultures of adult-rat hepatocytes*

Dose of aflatoxin (μg/culture)	No. of cells attached to culture dish (% of no. in controls)* in cultures with added	
	Aflatoxin B_1	Aflatoxin M_1
0.025	99 ± 5·7	116 ± 6·5
0·05	90 ± 10·5	118 ± 5·5
0·1	68 ± 6·9	105 ± 8·4
0·6	57 ± 5·5	79 ± 4·6
1·2	54 ± 5·1	55 ± 4·7
2·4	46 ± 5·1	58 ± 4·7

*Cells were plated at a density of $2·5 \times 10^6$ hepatocytes/60-mm diameter dish/4 ml culture medium. Aflatoxins were added to the hepatocytes 20–22 hr after isolation and the cultures were fixed and stained for counting 24 hr later.
Values are means ± SEM.

controls) and higher; however, doses between 0·1 and 0·6 μg/culture were not tested. Extrapolation from the data available suggests that a dose of AFM_1 as low as 0·2 μg/culture would have stimulated a release of LDH equivalent to that produced by 0·05 μg/culture of AFB_1. Use of the LDH assay as a measure of cytotoxicity was confirmed by observation of cell morphology and cell number by light microscopy. Cells exposed to high doses of either AFB_1 or AFM_1 had a more granular and vacuolated cytoplasm than controls. In addition, the monolayer was disrupted at high doses as indicated by the decreased number of cells attached to the dish (Table 1). Cytotoxicity resulting from either mycotoxin was delayed for at least 12 hr (data not shown), as previously reported for AFB_1 (Salocks *et al.* 1981).

DNA repair stimulated by AFB_1 and AFM_1

DNA repair was measured by two procedures, autoradiography (Fig. 2) and scintillation counting of TCA precipitates (Fig. 3). The same basic pattern was observed using either method. As in the assays for

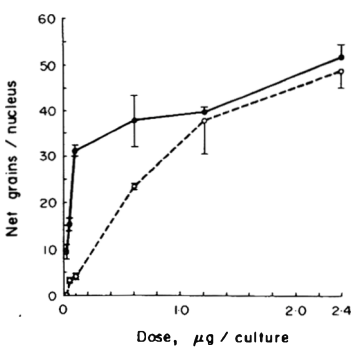

Fig. 2. Stimulation of DNA repair in primary cultures of rat hepatocytes by aflatoxin B_1 (●——●) and aflatoxin M_1 (○– –○)—analysis by autoradiography. Hepatocyte cultures ($2·5 \times 10^6$ cells/60-mm diameter dish/4 ml culture medium) were treated for 24 hr with aflatoxin B_1 or aflatoxin M_1. Each value is the mean of the average (net) grains/nucleus for three sets of 50 nuclei each, and the range bars indicate the SEM. The control grain count was −1·3 ± 0·4.

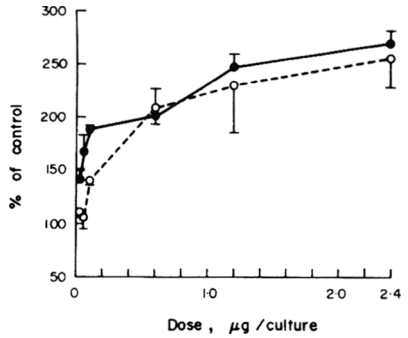

Fig. 3. Stimulation of DNA repair in primary cultures of rat hepatocytes by aflatoxin B_1 (●——●) and aflatoxin M_1 (○– –○)—analysis by liquid scintillation counting of trypsin-resistant TCA-precipitated material. Hepatocyte cultures ($2·5 \times 10^6$ cells/60-mm diameter dish/4 ml culture medium) were treated for 24 hr with aflatoxin B_1 or aflatoxin M_1. Data from two experiments has been pooled for this figure. Each point represents the mean ± SEM. Three cultures for each dose were used in each experiment.

cytotoxicity, the hepatocytes responded to lower doses of AFB_1 than AFM_1. The lowest dose of AFB_1 tested, 0·025 μg/dish, significantly ($P < 0·005$) stimulated DNA repair, as determined by autoradiography; 0·05 μg AFM_1/dish was required to produce a significant ($P < 0·005$) response in the analysis by liquid scintillation counting. Although a difference in potency was observed at the lower doses, AFB_1 and AFM_1 stimulated DNA repair to the same extent at the two highest doses. The fraction of cells undergoing DNA repair (Table 2) emphasized the potency of both aflatoxins. With four of the six AFB_1 doses, and three of six AFM_1 doses, about 100% of the cells had more than six grains per nucleus. Although the autoradiographic technique was slightly more sensitive for detecting unscheduled DNA synthesis, the method of scintillation counting of TCA precipitates readily detected DNA repair stimulated by these two chemicals.

Metabolism of AFB_1 and AFM_1

Although the metabolism of [^{14}C]AFB_1 by rat hepatocyte cultures has been reported previously (Decad *et al.* 1977; Decad *et al.* 1978; Salocks *et al.* 1981), data for [^{14}C]AFB_1 is also presented here to facilitate the comparison with [^{3}H]AFM_1 (Fig. 4). The metabolism of [^{3}H]AFM_1 was markedly slower than [^{14}C]AFB_1 metabolism (Fig. 4a,b). The level of chloroform-soluble radioactivity in cultures treated with AFB_1 declined rapidly (Fig. 4a). At the end of the 24-hr incubation period only 13% of the radioactivity could be extracted with chloroform. In [^{3}H]AFM_1-treated cultures, 26% of the radioactivity was chloroform-soluble after 24 hr. However, the AFM_1 metabolism curves (Fig. 4a) indicate that the decrease in chloroform-soluble radioactivity was greater than the decrease in parent compound. Since this discrepancy was probably due to a loss of tritium from [^{3}H]AFM_1 to water, the disappearance of the parent compounds with time provides a more accurate indication of the difference in metabolic rate. While no AFB_1 could be detected after 12 hr, 62% of the original dose of AFM_1 remained in the culture

Table 2. *Percentage of cells undergoing DNA repair in hepatocyte cultures treated with aflatoxin B_1 or aflatoxin M_1*

Dose of aflatoxin (μg/culture)	Percentage of cells with >6 (net) grains/nucleus* in cultures treated with	
	Aflatoxin B_1	Aflatoxin M_1
0 (control)	0	—
0·025	61	4
0·05	89	24
0·1	99	31
1·2	100	99
2·4	100	100

*After 20–22 hr in culture, rat hepatocytes were treated with aflatoxin B_1 or aflatoxin M_1 in propylene glycol and with [^{3}H]thymidine. After a further 24-hr incubation, the cells were processed for autoradiography. The number of grains in a nucleus-sized area of the cytoplasm was subtracted from the number of grains in the nucleus. For each dose, 150 nuclei were scored. Nuclei with >6 (net) grains were counted as a positive DNA-repair response.

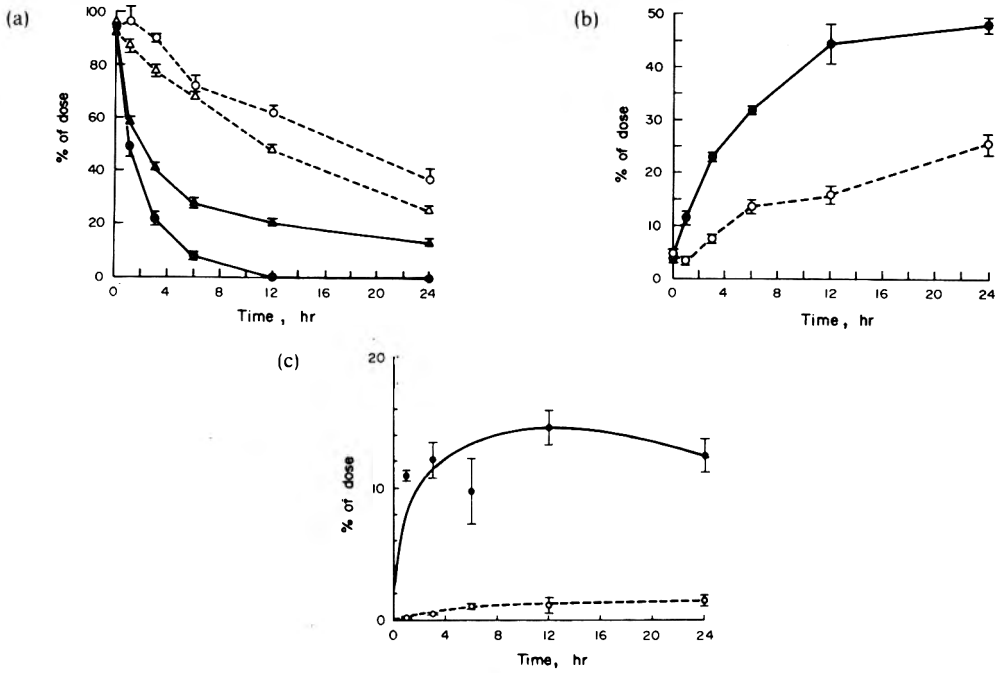

Fig. 4. [^{14}C]Aflatoxin B_1 ([^{14}C]AFB_1) and [^{3}H]aflatoxin M_1 ([^{3}H]AFM_1) disposition in primary hepatocyte cultures. A representative experiment is shown (n = 2). The dose of [^{14}C]AFB_1 was $5{\cdot}6 \times 10^6$ dpm/0·170 μg/culture. The dose of [^{3}H]AFM_1 was $1{\cdot}5 \times 10^6$ dpm/0·168 μg/culture. Each point represents the mean for three cultures and the range bars indicate the SEM. The average recovery of radioactivity was 88% for [^{14}C]AFB_1 and 93% for [^{3}H]AFM_1.

(a) Disappearance of aflatoxins from the culture medium of primary rat hepatocyte cultures. [^{14}C]AFB_1: radioactivity in chloroform phase (▲), unmetabolized AFB_1 in chloroform phase (●). [^{3}H]AFM_1: radioactivity in chloroform phase (△), unmetabolized AFM_1 in chloroform phase (○). Control cells were killed with 10% TCA prior to chemical treatment. After a 24-hr incubation, 90% of the dose of AFB_1 and 82% of the dose of AFM_1 was recovered as the parent compound in the chloroform extract of the control cell.

(b) Appearance of water-soluble metabolites of [^{14}C]AFB_1 and [^{3}H]AFM_1 in the culture medium of primary rat hepatocyte cultures treated with [^{14}C]AFB_1 (●) and [^{3}H]AFM_1 (○): radioactivity in the aqueous phase. The amount of radioactivity recovered in the aqueous phase from TCA-killed control cells treated with [^{14}C]AFB_1 or [^{3}H]AFM_1 was subtracted from values obtained with live cells.

(c) Covalent binding of [^{14}C]AFB_1 and [^{3}H]AFM_1 metabolites to hepatocyte macromolecules indicated by the amount of radioactivity strongly associated with TCA-precipitated material from cells treated with [^{14}C]AFB (●) and [^{3}H]AFM_1 (○). The amount of radioactivity associated with control cells killed with TCA prior to aflatoxin treatment was subtracted from the values obtained with live cells (0·2% of the dose for [^{3}H]AFM_1 and 1·5% for [^{14}C]AFB_1).

medium at that time. The production of polar metabolites from the mycotoxins was indicated by the increase in radioactivity in the aqueous phase (Fig. 4b). Compared to [^{3}H]AFM_1, at least twice as much [^{14}C]AFB_1 was converted to polar forms. From these data (Fig. 4a), it appears that 0·170 μg AFB_1/culture of hepatocytes was essentially all metabolized within 24 hr. An equivalent dose of AFM_1 was about 60% metabolized in that time period.

Both [^{14}C]AFB_1 and [^{3}H]AFM_1 were apparently metabolized to active forms which became bound to hepatocyte macromolecules. Figure 4c shows that approximately ten times as much AFB_1 as AFM_1 was strongly associated with TCA-precipitatable material after solvent extractions. At 24 hr, 12·5% of the dose of [^{14}C]AFB_1 was covalently bound compared to 1·5% of the dose of [^{3}H]AFM_1. The amount of cell material in cultures treated with the aflatoxins did not differ at the end of the 24-hr incubation (19·4 $\pm$ 1·4 μg DNA/AFB_1-treated culture *vs.* 21·4 $\pm$ 0 μg/AFM_1-treated culture). Even considering that AFB_1 was completely metabolized during the time course of the study and AFM_1 was not, AFB_1 was still covalently bound to a much greater extent.

The mean total recovery of added radioactivity was 88% for [^{14}C]AFB_1 and 93% for [^{3}H]AFM_1. The extraction procedure for [^{14}C]AFB_1 and [^{3}H]AFM_1 recovered 96 and 92% of the dose, respectively, in the chloroform phase of culture medium spiked with the radiolabelled compounds.

DISCUSSION

Both AFB_1 and AFM_1 were potent cytotoxins and genotoxins to cultured rat hepatocytes. Compared to other chemicals that have been tested in either of these two assays, the doses of the aflatoxins that produced a positive response were significantly lower. For example, the concentrations of the hepatotoxic chemical, acetaminophen, that caused the release of intracellular enzymes from cultured hepatocytes were approximately two orders of magnitude higher than those required to produce a toxic response by the aflatoxins in this experiment (Acosta *et al.* 1980). In the DNA-repair assay, AFB_1 is among the most potent chemicals to have been tested. The lowest concentration of AFB_1 that has previously been reported to produce a positive response in cultured hepatocytes was 10^{-5}M (Michalopoulos *et al.* 1978). The majority of genotoxic chemicals that have been tested were active in the range of 10^{-5}–10^{-3}M (Sirica & Pitot, 1980). In comparison, the lowest positive doses in the experiments reported here were 2×10^{-8}M-AFB_1 (0·025 μg/culture) and 4×10^{-8}M-AFM_1 (0·05 μg/culture).

This apparent increase in the sensitivity of the cytotoxicity and DNA-repair assays was probably due to the use of the hormone-supplemented serum-free culture medium formulated by Decad *et al.* (1977). Several investigators have found that this complex medium maintains the levels of foreign chemical metabolizing enzymes in cultured hepatocytes (Decad *et al.* 1977; Dickens & Peterson, 1980; Paine & Legg, 1978). In addition, recent studies (Sirica *et al.* 1980) suggest that a similar hormone supplemented culture medium also maintained the DNA-repair systems, independent of any effect on xenobiotic metabolism. Thus a more sensitive DNA-repair assay has resulted.

The metabolic fate of AFB_1 and AFM_1 in cultured hepatocytes correlated well with their activity in the cytotoxicity and DNA-repair assays at low doses, indicating that AFB_1 is probably the more toxic chemical. The dose chosen for the metabolism studies (0·17 μg/culture) was in the range on the cytotoxicity and DNA-repair dose-response curves where the maximum difference in AFB_1 and AFM_1 toxicity occurred. Much evidence indicates that AFB_1 requires metabolic activation with subsequent covalent binding to RNA, DNA and proteins in order to exhibit toxic, mutagenic and carcinogenic effects (Campbell & Hayes, 1976; Garner, Miller & Miller, 1972; Swenson, Lin, Miller & Miller, 1977; Wong & Hsieh, 1976). The same seems to be true for AFM_1 (Garner *et al.* 1972; Wong & Hsieh, 1976). Therefore, as predicted by these earlier studies and the hepatocyte cytotoxicity and genotoxicity data, metabolites of both AFB_1 and AFM_1 became covalently bound to macromolecules, but the level of AFB_1 binding was approximately ten times the level of AFM_1 binding (Fig. 4c). Similar differences in covalent binding were observed in protein of rat- and mouse-liver S-10 fractions incubated with AFB_1 and AFM_1 (Rice & Hsieh, 1981) and in DNA from livers of rats dosed *in vivo* with these two mycotoxins (Lutz, 1979). Since the hepatocyte cultures convert a large fraction of AFB_1 to AFM_1 (Decad *et al.* 1977), it is probable that some of the covalently bound residues in AFB_1-treated cells are due to activated AFM_1. In fact, AFM_1 adducts have been identified in the DNA of perfused liver dosed with AFB_1 (Essigmann, Donahue, Story, Wogan & Brunengraber, 1980).

In addition to the difference in the extent of covalent binding, the metabolism of AFM_1 was considerably slower than that of AFB_1. Approximately 50% of the dose of AFB_1 was absorbed and metabolized in 1 hr, while 18 hr were required for the same degree of AFM_1 metabolism. No AFB_1 could be detected at time points later than 6 hr, even though 20% of the total radioactivity was still recovered in the chloroform phase at 12 hr and 13% at 24 hr (Fig. 4a). Only AFM_1 was detected in the chloroform extracts from samples taken at 12 and 24 hr from cells originally treated with AFB_1 (data not shown). These data agree with the results of Decad *et al.* (1977), who found that AFM_1 accumulated in the culture medium of hepatocytes during the metabolism of AFB_1, reaching a maximum concentration around 6 hr after the addition of AFB_1. The significantly slower hepatic metabolism and slightly increased polarity of AFM_1 are probably responsible for its excretion as a urinary metabolite by several species (Campbell & Hayes, 1976; Wong & Hsieh, 1980).

In the low-dose range, AFB_1 stimulated more cytotoxicity and DNA repair in cultured hepatocytes than did AFM_1, but at higher doses (1·2–2·4 μg/culture), both mycotoxins produced equal responses in the two tests. One explanation for the plateau in DNA repair is that the repair enzymes have been saturated by high levels of damage. Saturation of repair systems has been demonstrated for other chemicals and ultraviolet light (Ahmed & Setlow, 1979). However, in our studies, cell death also plateaued, with approximately

50% of the cells remaining viable. Since both the DNA repair and cytotoxicity responses levelled off in the same dose range, it seems likely that the xenobiotic metabolizing enzymes are saturated by high doses of aflatoxin, and that approximately equal amounts of AFB_1 and AFM_1 activation are occurring, resulting in the same level of cytotoxicity and DNA repair. The saturation of carcinogen metabolism in cultured hepatocytes has been documented with high doses of 2-acetylaminofluorene (Spilman & Byard, 1981) and AFB_1 (G. M. Decad, D. P. H. Hsieh and J. L. Byard, unpublished data).

Caution is necessary when using the degree of stimulation of DNA repair to predict the relative carcinogenicity of chemicals, since DNA damage due to different carcinogens is repaired by the removal and resynthesis of DNA segments of varying length (Regan & Setlow, 1974). However, structurally related compounds such as the aflatoxins often produce similar patterns of DNA adducts. In fact, in recent studies it has been found that AFB_1 and AFM_1 both bind to the N-7 position of guanine (Essigmann, Croy, Nadzam *et al.* 1977; Essigmann *et al.* 1980). These adducts are removed from DNA rapidly, with similar half lives, probably by an enzymatic process rather than by spontaneous depurination (Croy & Wogan, 1981). Therefore, covalently bound forms of AFB_1 and AFM_1 are probably repaired by similar systems and the observed difference in the DNA-repair response of the hepatocytes to the two chemicals was due to the presence of more AFB_1 adducts in the DNA rather than the removal and resynthesis of longer DNA segments for each adduct.

Primary cultures of rat hepatocytes were a very sensitive indicator of aflatoxin-induced toxicity. The accumulated evidence from the present studies and previous ones on the comparative carcinogenicity of AFB_1 and AFM_1 agree—AFM_1 is less potent. At equal doses of the mycotoxins, AFM_1 causes fewer tumours in rainbow trout (Sinnhuber *et al.* 1974) and rats (Wogan & Paglialunga, 1974), is less mutagenic to *Salmonella typhimurium* (Wong & Hsieh, 1976), produces fewer DNA adducts in rat liver (Lutz, 1979), and stimulates less DNA repair in cultured rat hepatocytes. AFM_1 is probably a less active cytotoxin as well. Even though the biotransformation of AFB_1 to AFM_1 could be viewed as a detoxification step, very low doses of AFM_1 were active in the hepatocyte cytotoxicity and DNA-repair assays. Therefore, compared to AFB_1, the most potent chemical carcinogen known, AFM_1 is less toxic, but compared to other chemicals, AFM_1 is a potent hepatotoxin and carcinogen.

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IN VITRO METABOLISM OF PENICILLIC ACID WITH MOUSE-LIVER HOMOGENATE FRACTIONS

P. K. CHAN* and A. W. HAYES*

Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216, USA

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Abstract—The metaboism of penicillic acid (PA), a carcinogenic mycotoxin, was studied *in vitro* using subcellular fractions of mouse-liver homogenates. PA reacted with glutathione (GSH) both enzymatically and non-enzymatically. Each reaction was of equal importance. The *in vitro* metabolism of PA using different hepatic subcellular fractions was essentially non-enzymatic when GSH was absent, but metabolism was strikingly increased when GSH was available. In the microsomal preparation in the presence of GSH 75% of the added PA was biotransformed within 30 min to metabolite(s) that were not extractable with organic solvents. HPLC analysis indicated that the metabolite(s) were more polar than the parent compound.

INTRODUCTION

PA, a secondary metabolite produced by several food-borne fungi (Scott, 1978), is an α,β-unsaturated conjugated lactone (Fig. 1). PA has a variety of biological activities including antibacterial (Oxford, 1942), antiviral, antitumour (Suzuki, Kimura, Saito & Ando, 1971) and antidiuretic (Murnaghan, 1946) properties, digitalis-like action on cardiac muscle and dilating action on coronary and pulmonary vessels (Murnagham, 1946). PA also is cytotoxic (Umeda, 1971), hepatotoxic (Chan, Hayes, Meydrech & Ciegler, 1980a; Chan, Reddy & Hayes, 1980b; Ciegler, Mintzlaff, Wiesleder & Leistner, 1972; Kobayashi, Tsunoda & Tatsuno, 1971) and carcinogenic in mice and rats (Dickens & Jones, 1961, 1963 & 1965). The potential human health hazard of PA was suggested when it was isolated from blue-eye diseased corn, poultry feed, commercial corn, dried beans and tobacco products (Bacon, Sweeney, Robbins & Burdick, 1973; Kurtzmann & Ciegler, 1979; Pero, Harven, Owens & Snow, 1973; Snow, Lucas, Harvens & Owens, 1972; Thorpe & Johnson, 1974).

The mechanism of hepatotoxicity of PA has been investigated and the possible involvement of an active metabolite which might be detoxified by GSH proposed (Chan *et al.* 1980a). PA was most toxic by ip administration, followed by iv and oral administration (Chan, Phillips & Hayes, 1979; Murnaghan, 1946). The difference in the acute toxicity of PA when given by different routes of administration correlated well with the kinetic pattern of PA in mice, assuming that the liver was the site of bioactivation of PA (P. K. Chan, A. W. Hayes, M. Y. Siraj & E. F. Meydrech, unpublished data, 1981). Preliminary study of the *in vivo* metabolism of PA indicated that its major metabolites in both bile and urine were more polar than the parent compound, and that these metabolites were not extractable with organic solvents (P. K. Chan, A. W. Hayes & M. Y. Siraj, unpublished data, 1981). At least three of these metabolites were derived from cysteine or GSH. Since PA also can react with GSH non-enzymatically, two major detoxification pathways of PA involving GSH were proposed; an enzymatic and a non-enzymatic pathway (Chan *et al.* 1980a). In order to substantiate this hypothesis, an *in vitro* metabolism study of PA was conducted to examine each pathway and to define each quantitatively.

EXPERIMENTAL

Animals and materials. Male ICR mice (33–36 g), obtained from Charles River Mouse Farm (Wilmington, MA), were housed in an artificially illuminated (12 hr/day) and temperature-controlled (22 ± 1·1°C) room which was free from known sources of toxic contaminants. Standard laboratory rodent chow (Ralston Purina Co., Richmond, IN) and water were given *ad lib.*

Penicillic acid (PA) was produced as described by Chan *et al.* (1980b). Its purity (>99%) was established by ultraviolet absorption, melting point, and by TLC and HPLC. The radio-purity (>99%) and chemical purity (>99%) of ^{14}C-labelled PA, a gift from Dr A. Ciegler (United States Department of Agriculture, New Orleans, LA, USA), were confirmed by radiochromatography and HPLC, respectively. GSH was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and organic solvents used were of the highest purity available.

Non-enzymatic and enzymatic reaction of GSH with PA. The non-enzymatic reaction of GSH and PA was examined by incubating PA (0·1 M) in 2·5 ml of a mixture containing GSH, phosphate buffer (0·1 M, pH 7·4), KCl (27·7 mM), $MgCl_2$ (5·0 mM), $NADP^+$ (0·24 mM) and G6P (4 mM) at 37°C in 25-ml Erlenmeyer flasks.

*Present address: Toxicology Department, Rohm & Haas Co., 727 Norristown Road Spring House, PA 19477, USA.

Abbreviations: G6P = Glucose-6-phosphate; GSH = glutathione; HPLC = high-performance liquid chromatography; PA = penicillic acid; TLC = thin-layer chromatography.

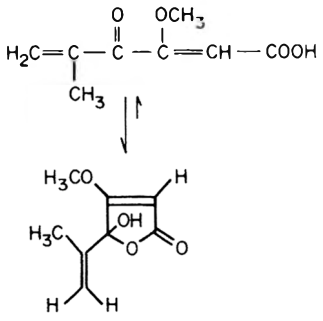

Fig. 1. Structure of penicillic acid.

The PA concentration in the incubation mixture was three times the concentration of GSH. The reaction was initiated by the addition of an aqueous solution of PA (200 μl of PA stock solution which gave a final PA concentration of 0·1 M). The incubation flasks were gently shaken during the 30-min incubation period. At 1, 2, 5, 10, 15, 20, 25 and 30 min after addition of PA, an aliquot (200 μl) of the mixture was acidified with 25% (w/v) aqueous *m*-phosphoric acid and extracted twice with three volumes of $CHCl_3$. The organic layers were separated, pooled and evaporated to dryness under nitrogen. The residue was then taken up in 100 μl of acetonitrile and an aliquot (10 μl) was analysed by HPLC for PA (Chan, Siraj & Hayes, 1980).

The enzymatic reaction of GSH and PA was examined in a similar manner but the reaction mixture contained in addition 0·45 ml of a 9000-**g** supernatant fluid from a 25% (w/v) liver homogenate. Non-specific protein binding and the GSH-independent enzymatic reaction of PA were examined as described above, except that GSH was omitted from the enzyme incubation mixture.

In vitro *metabolism of PA*. Male mice were killed by decapitation, and the livers were removed, weighed, rinsed with ice-cold 1·15% (w/v) KCl, blotted, cut into small pieces and homogenized in three volumes of 1·15% KCl. Aliquots of the whole homogenate were centrifuged at 600 **g** for 10 min to obtain the nuclei and cell-debris fraction. The 600-**g** supernatant fluid was centrifuged at 10,000 **g** for 30 min to obtain the mitochondrial fraction, and the supernatant fluid was centrifuged again at 105,000 **g** for 60 min to obtain the microsomal fraction. The resultant supernatant was taken as the cytosol fraction. Each fraction was resuspended in 1·15% KCl so that the final suspension contained approximately 20 mg of protein/ml for the nuclear and mitochondrial fractions, and 3 mg protein/ml for the microsomal fraction. Protein concentration was determined as described by Lowry, Rosebrough, Farr & Randall (1951).

The incubation mixture contained a subcellular fraction (3 mg/ml protein), phosphate buffer (0·1 M, pH 7·4), KCl (27·7 mM), $MgCl_2$ (5·0 mM), $NADP^+$ (0·8 mM), G6P (10 mM) and G6P-dehydrogenase (2·0 U/vessel). The mixture was equilibrated in a Dubnoff metabolic shaking incubator at 37°C for 1 min before initiating the reaction by adding 100 μl [^{14}C]PA solution (10,000 cpm/vessel). The final concentration of PA was 0·1 M. After a 30-min incubation at 37°C, the reaction was stopped by adding 100 μl of ice-cold *m*-phosphoric acid (25%). The resulting mixture was extracted twice with ethyl acetate and then with ethanol–ether (1:3, v/v). The organic layers were separated, pooled and concentrated by evaporation under nitrogen. Ten millilitres of scintillation cocktail (PCS; Amersham, Arlington Heights, IL) were added to the ethyl acetate, ethanol–ether and aqueous extracts for liquid scintillation spectrometry.

Reverse phase HPLC was performed to separate the PA metabolites. A Waters Associates (Milford, MA) HPLC system with a pre-column, a guard column (Whatman, Inc., Clifton, NJ) and a C-18 μBondapak analytical column was used, with detection at 254 nm. The elution system consisted of distilled-in-glass acetonitrile (Burdick and Jackson Laboratories, Muskegon, MI), glass-distilled water and acetic acid (Fisher Scientific Co., Fairlawn, NJ). The system was optimized on the basis of sensitivity and separation of PA or its metabolite(s) from interfering substances. The following system was used: mobile phase, acetonitrile–distilled water–0·5% (v/v) acetic acid (25:75:0·3, by vol.); flow rate, 1·2 ml/min. For HPLC analysis, the ethyl acetate and aqueous extracts were evaporated to dryness under nitrogen. The ethyl acetate and aqueous residues were taken up in 100 μl of acetonitrile or mobile solvent, respectively. A 25-μl aliquot was injected into the HPLC. HPLC column eluates were collected at 10-sec intervals and 10 ml of PCS were added to each for liquid scintillation spectrometry. Quenching was corrected by external standard quench correction. The lag time between ultraviolet detection and collection of radioactivity fractions was determined by injecting 10 μg of non-labelled pure PA into the HPLC and collecting eluate fractions at 5-sec intervals. Aliquots (10 μl) of each fraction collected were re-injected into the HPLC for analysis of PA concentration. The lag time was calculated by subtracting the PA retention time from the retention time corresponding to the eluate fraction with the highest PA concentration. Radioactivity profiles of column eluates were corrected for the estimated lag time.

RESULTS

The results of both enzymatic (using 9000-**g** liver homogenate supernatant fluid as the enzyme source) and non-enzymatic reactions of PA with GSH are shown in Fig. 2. PA was stable in the incubation mixture in the absence of either the enzyme or GSH. Almost all (>98%) of the added PA was recovered as the unchanged form over the 30-min incubation period. Approximately 20% of the PA reacted with GSH non-enzymatically during the 30-min incubation period. After correcting for protein binding and GSH-independent enzymatic reaction(s), the total GSH-dependent reaction (enzymatic and non-enzymatic) could be attributed to 40% of the original dose while 20% of this could be attributed to the GSH-dependent enzymatic reaction.

The metabolism of PA in the presence of different liver homogenate fractions is shown in Table 1. Most of the ethyl acetate extractable radioactivity could be

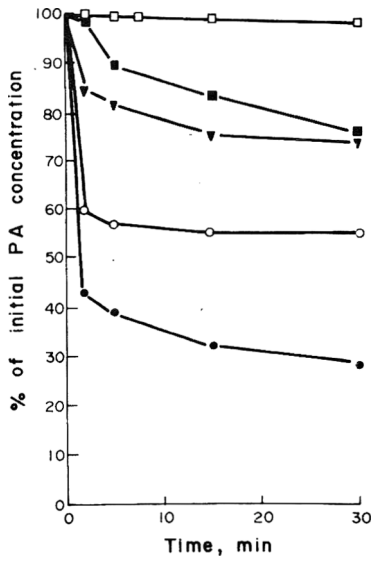

Fig. 2. Enzymatic and nonenzymatic reactions of penicillic acid (PA) with glutathione (GSH). A 9000-g supernatant fluid of liver homogenate was used as the enzyme source. To the basic incubation mixture (see text) was added PA only (□), PA + GSH (■), PA + enzyme (▼) or PA + GSH + enzyme (●). After correcting for protein binding and GSH-independent enzymatic reactions, the total GSH-dependent reaction is shown (○). The PA concentration was determined by HPLC.

attributed to authentic PA. Microsomal metabolism of PA yielded about twice the concentration of non-extractable (not extracted by either ethyl acetate or ether–ethanol) metabolites in the aqueous phase than did the nuclear or mitochondrial fractions. However, an even larger concentration of non-extractable PA metabolites was formed in the cytosol fraction (approximately five times that formed in the microsomal fraction). Radioactivity extractable with ether–ethanol (3:1, v/v) accounted for less than 1% of the initial dose in any of the subcellular fractions. When GSH was included in the microsomal incubation mixture, most of the radioactivity derived from [^{14}C]PA was not extracted into either ethyl acetate or ether–ethanol (3:1, v/v), and the amount of non-extractable metabolites in the aqueous phase increased from 3 to 75% of the initial dose. Little change was observed in the amount of non-extractable metabolites formed when heat-treated enzyme was used. However, the amount of ethyl-acetate extractable derivatives, including PA itself, decreased. This probably was due to an increase in PA binding to denatured protein. A small decrease in the amount of non-extractable metabolites formed was observed in the incubation mixtures without the NADPH generating system (Table 1).

The HPLC chromatogram tracings and radioactivity profiles of column eluates of extracts from the *in vitro* metabolism of [^{14}C]PA with whole liver homogenate are shown in Fig. 3. A small radioactivity peak (retention time of 130 sec) of the ethyl acetate-extractable metabolite(s) was observed (Fig. 3a,b). At least three radioactivity peaks and at least two chromatogram peaks (A and B) of non-extractable PA metabolites were identified, respectively, from the radioactivity profiles of column eluates and the HPLC chromatogram tracings (Fig. 3c,d). These

Table 1. *Subcellular distribution and co-factor requirements of penicillic acid metabolism* in vitro

Liver fraction and treatment	Recovery (%) of radioactivity (administered as [^{14}C]penicillic acid) in		
	Ethyl acetate extracts	Ether–ethanol (3:1, v/v) extract	Aqueous layer
Whole homogenate			
Untreated	83·95 ± 0·98 (91·32)	1·02 ± 0·19	9·04 ± 0·48
Heat-treated	76·63 ± 0·98 (94·00)	0·6 ± 0·03	8·33 ± 1·28
Without NADP$^+$	80·10 ± 1·10 (72·18)	0·83 ± 0·13	8·45 ± 0·1
Nuclear			
Untreated	91·11 ± 1·58 (95·36)	0·72 ± 0·26	1·92 ± 0·09
Heat-treated	80·22 ± 0·55 (95·80)	0·84 ± 0·08	2·21 ± 0·58
Without NADP$^+$	91·71 ± 0·35 (97·60)	0·66 ± 0·10	1·63 ± 0·75
Mitochondrial			
Untreated	93·92 ± 1·60 (94·58)	0·93 ± 0·21	1·66 ± 0·09
Heat-treated	89·05 ± 2·47 (90·84)	0·45 ± 0·20	2·65 ± 0·81
Without NADP$^+$	90·95 ± 1·18 (92·70)	0·73 ± 0·09	1·32 ± 0·66
Microsomal			
Untreated	91·89 ± 0·87 (97·08)	0·49 ± 0·12	3·22 ± 0·125
Heat-treated	87·13 ± 1·13 (94·64)	0·83 ± 0·11	3·07 ± 0·24
Without NADP$^+$	91·14 ± 0·36 (92·89)	0·54 ± 0·2	2·69 ± 0·22
With added glutathione	14·29 ± 0·37 (87·68)	0·34 ± 0·22	74·87 ± 1·2
Cytosol	71·24 ± 0·61 (95·04)	0·69 ± 0·09	15·6 ± 0·76
Heat-treated	63·68 ± 1·38 (96·93)	1·05 ± 0·15	16·05 ± 0·70
Without NADP$^+$	73·09 ± 1·39 (89·28)	0·42 ± 0·10	13·02 ± 0·78

Values are means ± SEM for three determinations. The values in brackets show the percentage of radioactivity recovered as authentic penicillic acid.

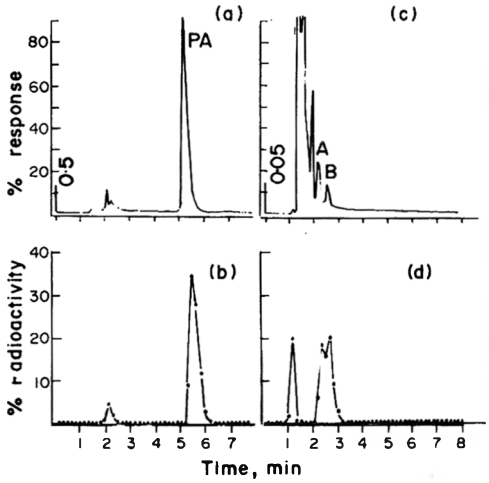

Fig. 3. HPLC chromatogram tracings and radioactivity profiles of column eluates of (a,b) ethyl acetate extracts and (c, d) aqueous extracts from the metabolism *in vitro* of [^{14}C]penicillic acid (PA) by whole liver homogenates. For details of HPLC system, see Experimental.

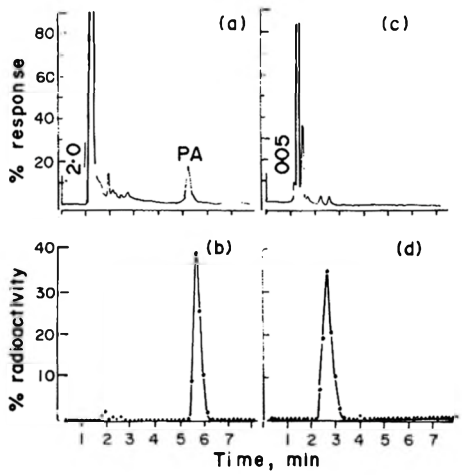

Fig. 5. HPLC chromatogram tracings and radioactivity profiles of column eluates of (a, b) ethyl acetate extracts and (c, d) aqueous extracts from the *in vitro* microsomal metabolism of [^{14}C]penicillic acid in the presence of glutathione. For details of the HPLC system, see Experimental.

could be attributed to more polar metabolites of the parent compound which was eluted at 305 sec in the HPLC system used.

Only a very small radioactivity peak of a polar metabolite(s), extracted by ethyl acetate from the microsomal incubation mixture, was noted (Fig. 4a,b). However, at least three radioactivity peaks and three corresponding chromatogram peaks of non-extractable metabolites in the aqueous extract of the cytosol incubation mixture were identified in the radioactivity profile and HPLC chromatogram tracing, respectively (Fig. 4c,d).

When GSH was included in the microsomal incubation mixture, the amount of PA extracted by the ethyl acetate was drastically decreased (Fig. 4a,b; Fig. 5a,b). Several small radioactivity peaks were still visible in the radioactivity profile between 90 and 180 sec (Fig. 5b). This decrease in PA extracted into ethyl acetate was accompanied by a parallel increase of polar metabolites in the aqueous extract (Fig. 5c,d). Two chromatogram peaks at 140 and 160 sec and one radioactivity peak at 160 sec corresponding to non-extractable polar metabolites of PA in the aqueous extract were noted, respectively, from the HPLC chromatograph tracings and the radioactivity profile (Fig. 5c,d).

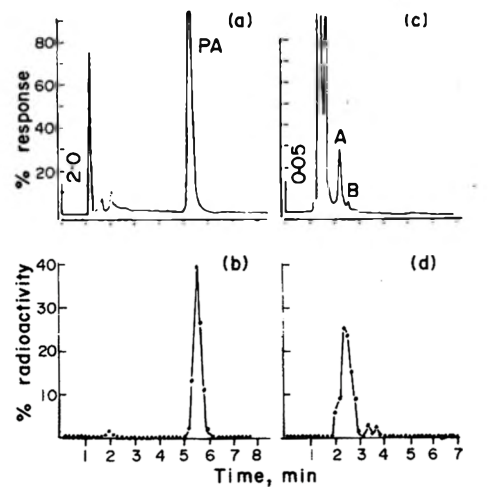

Fig. 4. The metabolism *in vitro* of [^{14}C]penicillic acid by microsomal and cytosol fractions of liver homogenates. HPLC chromatogram tracings and radioactivity profiles of column eluates of (a, b) ethyl acetate extracts of microsomal incubations and (c, d) aqueous extracts of cytosol incubations. For details of the HPLC system, see Experimental.

DISCUSSION

Non-enzymatic reactions of PA with compounds such as GSH that contain a free sulphydryl group have been reported to occur (Black, 1966; Ciegler *et al.* 1972; Geiger & Conn, 1945; Lieu & Bullerman, 1978; Nakamura, Ohta & Ueno, 1977; Rinderknecht, Ward, Bergel & Morrison, 1947). However the enzymatic reaction of PA with GSH has not been investigated. This report is the first demonstration of an enzymatic reaction of PA with GSH. It has significant bearing on the proposed mechanism of PA-induced hepatotoxicity (Chan *et al.* 1980a). The toxicity of PA was greater in mice pretreated with phenobarbital (an enzyme inducer) than in non-pretreated mice but GSH depletion by PA did not increase in phenobarbital pretreated animals (Chan *et al.* 1980a). It is possible that PA is detoxified *via* two equally important pathways, a non-enzymatic reaction and a reaction through enzymatic activation of PA. The active metabolite is subsequently detoxified by GSH. Increasing the activation pathway would not only increase toxicity by increasing the rate of covalent binding to macromolecules but also increase depletion of hepatic GSH *via* this pathway. Assuming that all reaction rates follow first-order kinetics, this increase in GSH depletion *via* an enzymatic pathway would

decrease the parent compound available for the non-enzymatic reaction, resulting in no net change in PA-induced GSH depletion in phenobarbital-treated animals. The fact that in the present study equal amounts of PA were found to react with GSH *via* enzymatic and non-enzymatic pathways further supports the suggestion that GSH-dependent enzymatic and non-enzymatic reactions are of equal importance in detoxifying PA. However, whether or not both reactions occur *in vivo* is not known.

Most of the added toxin was recovered unchanged when GSH was not present in the incubation mixture. However, a small amount of non-extractable polar metabolites was found in the aqueous phase. These metabolites probably were the PA conjugates of endogenous cysteine or GSH. The small amount of polar metabolite(s) extracted by ethyl acetate might be glucuronide conjugates of PA. Glucuronide conjugates of PA or its metabolites were found *in vivo* (P. K. Chan, A. W. Hayes, M. Y. Siraj, unpublished data, 1981; Park, Friedman & Heath, 1980).

In comparison with the nuclear, mitochondrial and microsomal fractions, considerably larger amounts of non-extractable metabolites were found in the cytosol and the whole homogenate fractions in which most of the endogenous GSH is located. When GSH was added to the complete microsomal incubation mixture containing $NADP^+$, 75% of the added toxin was transformed into non-extractable metabolite(s). These results indicated that GSH or cysteine was the limiting factor in the *in vitro* metabolism of PA. Non-enzymatic reactions probably were responsible for the small amount of metabolite(s) found in each fraction because there were only small differences between the heat-treated and non-heat-treated fractions in the amounts of metabolites formed.

The chemical structures of GSH and PA reaction products, via either the enzymatic or non-enzymatic reaction, have not been identified. Elucidation of product structures would help support or negate the hypothesis of two GSH-dependent metabolic pathways for PA.

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USEFULNESS AND LIMITATIONS OF VARIOUS GUINEA-PIG TEST METHODS IN DETECTING HUMAN SKIN SENSITIZERS—VALIDATION OF GUINEA-PIG TESTS FOR SKIN HYPERSENSITIVITY

F. MARZULLI*

Food and Drug Administration, Washington, DC

and

H. C. MAGUIRE, JR

Hahnemann Medical College and Hospital, Philadelphia, PA 19102, USA

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Abstract—Several guinea-pig predictive test methods were evaluated by comparison of results with those obtained with human predictive tests, using ten compounds that have been used in cosmetics. The method involves the statistical analysis of the frequency with which guinea-pig tests agree with the findings of tests in humans. In addition, the frequencies of false positive and false negative predictive findings are considered and statistically analysed. The results clearly demonstrate the superiority of adjuvant tests (complete Freund's adjuvant) in determining skin sensitizers and the overall superiority of the guinea-pig maximization test in providing results similar to those obtained by human testing. A procedure is suggested for utilizing adjuvant and non-adjuvant test methods for characterizing compounds as of weak, moderate or strong sensitizing potential.

INTRODUCTION

During the past score of years, in the USA, there has been a proliferation of Federal regulatory agencies. This has led to increasing requirements for tests on animals and humans which are intended to characterize the potential toxicity of a wide variety of personal products, drugs, pesticides and other chemicals with which humans may come into contact. Ethical considerations require that laboratory animals be used for this purpose rather than humans. Monetary, political and time considerations would favour the elimination of the use of both humans and animals and reliance instead on *in vitro* tests. In some cases this latter approach is achievable. However, in the area of skin sensitization, tests for contact-allergy potential require the use of intact mammals with functioning immunological systems.

Traditionally the animal species of choice for the conduct of skin hypersensitivity tests has been the guinea-pig. The first useful test was that devised by Draize, Woodard & Calvery (1944), based on observations reported by Landsteiner & Jacobs (1935). Candidate drugs and cosmetics were administered to guinea-pigs by intradermal injection in a prescribed manner (details of which follow) to ascertain their potential for human sensitization under conditions of use.

Subsequent variations in the basic procedure have included topical application to the skin without (Hood, Neher, Reinke & Zapp, 1965; Klecak, Geleick & Frey, 1977) and with (Buehler, 1965) occlusion of the skin test site. A significant development in prospective testing in guinea-pigs occurred with the introduction of complete Freund's adjuvant as a means of enhancing the skin sensitization potential (Landsteiner & Chase, 1940; Magnusson & Kligman, 1969).

To date, a number of prospective testing methods have been developed and widely touted as superior to the original Draize technique and yet we have limited information as to their usefulness in predicting skin sensitization potential in humans. One attempt to compare various guinea-pig assays was made by Prince & Prince (1977). They concluded that, in general, CFA (adjuvant) techniques were clearly superior to non-adjuvant techniques in detecting weak contact sensitizers. Magnusson & Kligman (1970) also reached a similar conclusion. However, there remains the separate problem of the applicability of test results in guinea-pigs to conditions of use by humans and to the results of testing in humans.

The present work is an attempt to predict by animal testing the effects of a compound when used by humans. While recognizing that predictive tests in humans have deficiencies in forecasting the sensitization potential of a chemical "in the field", this investigation is a rather large-scale comparison of test results obtained in guinea-pigs and in humans. In the

*Present address: National Academy of Sciences, National Research Council, Assembly of Life Sciences, 2101 Constitution Avenue, Washington, DC 20418, USA.

Abbreviations: CFA = Complete Freund's adjuvant; DMSO = dimethylsulphoxide; GPMT = guinea-pig maximization test.

main study, five guinea-pig predictive test methods have been used to evaluate 11 possibly allergenic substances that have been used in the cosmetics industry; the results have been compared with the findings of predictive tests in humans. In a subsidiary small study three additional test methods have been evaluated, using five of the 11 original test materials. Experience in humans suggested that most of the 11 chemicals were weak or negligible sensitizers. In addition, the allergenicity of 10 of the 11 chemicals had been evaluated by prospective testing in humans and the resultant sensitization rates were available for comparison. In the initial and major phase of this investigation, each of the 11 chemicals was subjected simultaneously to five different testing techniques: (1) the guinea-pig Draize method, (2) the Buehler method, (3) the guinea-pig maximization test of Magnusson and Kligman, (4) a strictly epicutaneous (split-adjuvant) test that we devised some years ago, and (5) a test that utilizes immunopotentiation with cyclophosphamide (Hunziker, 1968; Maguire, 1973; Maguire & Ettore, 1967).

The last three tests make use of CFA; the first two do not. A further investigation was undertaken to supplement the main body of work. This involved a limited evaluation of three non-occlusive, non-CFA techniques, having as reference the standard guinea-pig Draize test and the GPMT, using five of the original 11 compounds. In all of the work, the basic approach was to investigate each compound simultaneously with different techniques.

The results point out the merits and limitations of each testing technique as it relates to the evaluation of compounds of weak allergenicity, and suggest ways for improving the reliability of methods for identifying and ranking in the guinea-pig weak contact allergens of man.

EXPERIMENTAL

Guinea-pigs. Female Hartley-strain guinea-pigs weighing about 350 g at the time of purchase were obtained from commercial sources. The guinea-pigs were housed in metal cages on wood shavings in temperature-controlled (65 ± 5 C) rooms with a 12-hr (7 a.m.–7 p.m.) light cycle. They were fed fresh Purina guinea-pig chow and had constant access to tap water. Water delivery was by demand from tubes external to the guinea-pig cages.

Chemicals. Cyclophosphamide was purchased as a powder from Sigma Chemical Co., (St. Louis, MO) and diluted in pyrogen-free saline immediately prior to use. CFA (0638-60; Difco Laboratories, Detroit, MI) contained 0·5 mg heat-killed tubercle bacilli (*Mycobacterium butyricum*)/ml in a vehicle consisting of sterile light mineral oil and Arlacel A. The sources of the compounds tested were as follows: DMSO—Crown Zellerbach Corp., Chemical Products Division, Camas, Washington. DC; ethanol—Pharm Co., Publicker Industries Co., Linfield, PA; dimethyl citraconate (12% v/v)—lot no. AE 10393, supplied by the Food and Drug Administration, Washington. DC; methyl crotonate (6% v/v)—lot no. J8009, supplied by the Food and Drug Administration; Germall 115 [imidazolidinyl urea, 10% (v/v)]—Sutton Laboratories Inc., Roselle, NJ; formalin [37% (v/v) aqueous formaldehyde]—J. T. Baker Chemical Co., Phillipsburg, NJ; captan [1% (w/v); Vancide no. 898E]—R. T. Vanderbilt Co., Inc., Norwalk, CT; Dowicil 200 [quaternium-15, 5% (v/v)]—lot no. 01138005, Dow Chemical Co., Coral Gables, FL; hydroxycitronellal (20% v/v)—Fritzche-Dodge and Olcott, Inc., New York; vetivert acetate (20% v/v)—Givaudan Corp., Clifton, NJ; methyl methacrylate (10% v/v)—Eastman Kodak Co., Rochester, NY.

Sensitization techniques (Klecak, 1977)

Draize guinea-pig technique. A concentration of 0·1% (w/v for solids, v/v for liquids) test compound in saline was used throughout. In each round of testing, ten guinea-pigs were clipped on the left flanks and injected intradermally with 0·05 ml of test solution as their initial sensitizing exposure. The injection site was examined 24 hr later and the reactions measured and recorded. Nine subsequent intradermal injections were made, each time with a volume of 0·1 ml; a new site on the left flank was used each time. These injections were given three times per week. Two weeks after the last (10th) injection, the guinea-pigs were challenged in a clipped site on the opposite flank with 0·05 ml of test solution given intradermally. A similar injection was made in 10 control guinea-pigs. The reactions (at 24 hr) of the first (induction) and final (challenge) injections in the experimental animals, as well as the 24-hr reactions of the naïve control guinea-pigs, were compared in order to determine whether a change in reactivity to test material had taken place as a result of the sensitization procedure.

Buehler guinea-pig assay. The vehicle and the concentration of the test material are shown for each compound in Table 1. Ten guinea-pigs were clipped on the left flank; 0·5 ml of test compound was applied to the area and covered by Blenderm (3M Corp., Minneapolis, MN) and fixed in place by an encircling bandage of Elastoplast (Beiersodorf Inc., South Norwalk, CT). This occlusive bandage was secured by adhesive tape at either end; it was removed after 6 hr. The application was repeated 7 and 14 days later. On day 28 an area on the right flank was clipped and 0·5 ml of test compound was applied using the same technique as for the left flank except that Scanpore tape (Norgesplaster, A/S, Norway), which was less irritating, was substituted for regular adhesive tape. The dressing was removed after 24 hr. Ten control guinea-pigs were challenged in parallel. All reactions were read in experimental and control guinea-pigs 24 and 48 hr later and comparisons were made between groups.

(3) *Guinea-pig maximization test of Magnusson & Kligman.* Ten guinea-pigs were clipped in the mid-dorsal region near the scapula. Three sets of two intradermal injections (0·1 ml each) were given to each animal as follows: (1) CFA emulsified with an equal volume of sterile distilled water; (2) a 5% (liquids v/v; solids w/v) concentration of test compound in saline, (3) a 10% (liquids v/v; solids w/v) concentration of test compound in saline emulsified with an equal volume of CFA. On day 6 the injected sites were inspected and if there was no significant irritation at that time, 5% sodium lauryl sulphate was applied; if there was significant irritation, no sodium lauryl sulphate was applied to the site. On day 7, the area of the injections

Table 1. *Results of three rounds of testing of 11 compounds using five guinea-pig bioassays for allergenicity*

Compound	Concn and vehicle†	Test	No. of guinea-pigs with reaction*			
			Round 1	Round 2	Round 3	Cumulative
DMSO	—‡	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	0	0	0	0
		GPMT	0	0	0	0
		Split adjuvant	0	0	0	0
Ethanol	—‡	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	0	0	0	0
		GPMT	0	0	0	0
		Split adjuvant	0	0	0	0
Hydroxycit-ronellal	20% (v/w) in petrolatum	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	2	0	2	4
		GPMT	2	4	2	8
		Split adjuvant	2	2	1	5
Methyl-methacrylate	10% (v/v) in 100% ethanol	Draize	0	2	0	2
		Buehler	0	0	0	0
		Cy/CFA	0	0	0	0
		GPMT	0	0	0	0
		Split adjuvant	0	0	0	0
Captan	1% (w/w) in petrolatum	Draize	10	6	10	26
		Buehler	0	1	0	1
		Cy/CFA	8 (8)	10 (12)	10	28
		GPMT	9	7 (9)	8	24 (29)
		Split adjuvant	7	6	8	21
Formalin	5% (v/v) in saline	Draize	6	1	3	10
		Buehler	0	0	0	0
		Cy/CFA	4 (8)	0	0	4 (28)
		GPMT	2 (8)	1	2	5 (28)
		Split adjuvant	2	0	0	2
Dowicil 200	5% (w/w) in petrolatum	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	1	3	5	9
		GPMT	9	2	5 (9)	16 (29)
		Split adjuvant	4	3	4	11
Methyl crotonate	6% (v/w) in petrolatum§	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	1	1	0	2
		GPMT	2	3	1	6
		Split adjuvant	3	1	0	4
Vetivert acetate	20% (w/w) in petrolatum§	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	1	2	0 (9)	3 (29)
		GPMT	2	5	4	11
		Split adjuvant	6	2	2	10
Dimethyl citraconate	12% (v/w) in petrolatum	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	0	0	0	0
		GPMT	0	0	0	0
		Split adjuvant	1	0	1	2
Germall 115	10% (w/w) in petrolatum§	Draize	0	0	0	0
		Buehler	0	0	0	0
		Cy/CFA	0	3	0	3
		GPMT	0	2	1	3
		Split adjuvant	0	0	0	0

Cy/CFA = Cyclophosphamide/complete Freund's adjuvant bioassay
GPMT = Guinea-pig maximization test

*Ten guinea-pigs were used in each round (cumulative number = 30) except where the numbers used are indicated in brackets.

†For tests other than the Draize test, for which a concentration of 0·1% test compound was used throughout.

‡Except for the Draize test, induction with ethanol–DMSO (50:50, v/v) and a separate challenge with either 100% ethanol or 100% DMSO.

§Challenge with 25% test compound in Round 2 (except in the Draize test).

was clipped and 0·5 ml of the test compound (the concentration and vehicle are specified for each chemical in Table 1) was applied to a 1-in. square of Whatman No. 2 filter paper. The paper was positioned face down on the induction site, and sealed in place with Blenderm, Elastoplast and adhesive tape as in the Buehler wrappings. A challenge was made on day 21 with 0·5 ml of test material at a high non-irritant concentration (see Table 1). Application was made to a clipped site on the left flank using Whatman No. 2 filter paper, and a sealing of Blenderm, Elastoplast and Scanpore adhesive tape. The dressing was removed 24 hr later and readings were made 1 and 2 days later (i.e. 48 and 72 hr after the challenge).

Split-adjuvant technique. The concentration and vehicle for each test compound are shown in Table 1. Ten guinea-pigs were clipped on the right anterior flank, the area was shaved to the glistening layer with a razor blade and dry ice applied for 10 sec with firm pressure. A dressing containing a window was placed over the clipped, shaved, frozen site and 0·2 ml of test material was applied to the induction site and covered with Blenderm tape. The dressing was held in place with adhesive tape. Two days later 0·2 ml of test material was applied to the area sealed. On day 4, two injections of 0·075 ml CFA were made adjacent to the induction site, and this was followed by application of 0·2 ml of test material on the induction site. The site was resealed and on day 7 a further 0·2 ml of test material was applied through the window. On day 9 the dressing was removed. A challenge was made on day 22 using the GPMT method.

Cyclophosphamide/CFA bioassay. The concentration and vehicle for each test compound are shown in Table 1. Three days prior to induction (i.e. on day −3), 10 guinea-pigs were weighed and given by ip injection 150 mg cyclophosphamide/kg body weight. On day 0 the animals were clipped on the right anterior flank and dry ice was applied to the induction area for 5 sec. A dressing containing a window was applied as in the split-adjuvant technique (see above) and 0·2 ml of test material was placed on a 1-in. square of Whatman No. 2 filter paper and applied to the induction site through the window. This was sealed with Blenderm and adhesive tape. On each of days 1, 2, 3 and 4 the window was opened, 0·2 ml of the test material applied and the window re-sealed. On the afternoon of day 4 the dressing was removed and two intradermal injections (0·075 ml each) were made with CFA immediately adjacent to the induction site. On day 9, a 6-hr application of allergen to the induction site was made under occlusion. The guinea-pigs were challenged on day 22 using the GPMT technique.

Altered Draize technique. Preliminary tests were carried out on each chemical (in saline) in order to identify the highest concentration of chemical that failed to give a significant irritation reaction at 24 hr when 0·1 ml was injected intradermally on the flank. The concentration subsequently used for all five compounds tested was 0·5% (v/v for liquids; w/v for solids). For induction, 10 guinea-pigs were clipped as for the standard Draize test. They were injected on day 0 and three times weekly for a total of 10 injections, with 0·1 ml of the test solution. A fresh adjacent site was used for each successive injection. A challenge was made 2 wk after the last inducing injection with 0·1 ml of the test solution, as in the standard Draize test, using a freshly clipped site on the right flank. Control animals were challenged in parallel. The reactions were read at 24 hr and comparisons were made as in the standard Draize test.

Non-occlusive topical test. Ten guinea-pigs were clipped on the left flank. The test material (0·5 ml, concentration and vehicle as in Table 4) was applied to a 1 $in.^2$ area and the material was massaged gently into the skin for a few seconds. This was repeated daily 5 days/wk, i.e. for a total of 20 applications. The application site was clipped three times per week so as to maintain close contact between the test material and the surface epidermis. The animals were left untreated for 10 days after the last inducing application and then a challenge was made to a freshly clipped site on the opposite flank, using a fixed concentration of test materials (as shown in Table 4). The reactions were recorded after 24 and 48 hr. Control animals were tested in parallel and appropriate comparisons made. The sensitization and challenge schedule is similar to that of the open-epicutaneous test (Klecak *et al.* 1977). However, the latter makes use of a number of different concentrations for induction and challenge, whereas in this work fixed concentrations of test materials have been used.

Non-occlusive topical test using DMSO. The non-occlusive topical test (see above) was modified by pretreating the induction site with 0·02 ml of 100% DMSO 10–15 min before the application of the test material (concentration and vehicle as in Table 4). DMSO was not used at challenge (Maguire, 1974).

RESULTS

Results of three rounds (replicates) of tests (10 animals/round) with 11 compounds, using five guinea-pig assays for allergenicity are shown in Table 1. For the purpose of comparison, Table 2 shows results obtained when ten of these chemicals were tested on humans using the human Draize* predictive tests for delayed skin hypersensitivity (modified by using a high induction concentration of test material; Marzulli & Maibach, 1980).

Two types of comparison between these results are possible, one quantitative and one qualitative. As will be seen, the quantitative approach is less useful.

Quantitative assessment

Coefficients of correlation (R) between the response fractions obtained by each guinea-pig test and the response fraction obtained by the human test were estimated†. The results were: Draize 0·69, GPMT 0·65, cyclophosphamide/CFA 0·51, split adjuvant 0·41, Buehler 0·24. A positive correlation between guinea-pig and human findings was obtained in all cases except with the Buehler guinea-pig technique. These

*Note that there is a Draize guinea-pig test and a completely different Draize human test for skin sensitization. The test species (human or guinea-pig) is mentioned in connection with a 'Draize' test.

†Logarithmic transformations were involved. Rates were transformed to their square roots. This gives skewed results, nevertheless this transformation tends to stabilize variances.

Table 2. *Results of prospective testing (modified Draize test) in humans*

Compound	Concentration (%) used for		Reactions
	Induction	Challenge	
Ethanol	100	100	0/94
Hydroxycitronellal	20	20	1/99
Methyl methacrylate	10	10	0/184
Captan	1	1	9/205
Formalin	5	1	4/52
Dowicil 200	5	5	1/183
Methyl crotonate	12	12	1/99
Vetivert acetate	20	2	1/62
Dimethyl citraconate	12	12	0/104
Germall 115	10	10	1/184

positive associates are only weakly supportive of a quantitative relationship between guinea-pig and human test results; a high sensitization index in guinea-pigs does not necessarily indicate a similarly high sensitization index in humans.

Qualitative assessment

In the qualitative assessment, a substance was considered allergenic if one or more subjects showed a positive response in any test round (guinea-pigs) or group (humans). Otherwise, it was considered a negative finding for allergenicity. In this qualitative assessment a positive response rate of 1/100 has the same thrust as a rate of 9/10, both being considered an allergenic finding. Comparisons between guinea-pig test results are shown in Table 3. They show (a) the frequency with which guinea-pig and human tests results agree, (b) the frequency with which the guinea-pig test is falsely positive and (c) the frequency with which the guinea-pig test is falsely negative.

The adjuvant techniques (GPMT, Cy/CFA and split-adjuvant) show significantly (by chi-square test) greater agreement with human predictive findings than the non-adjuvant techniques (Draize & Buehler). On comparing the adjuvant techniques one with another, however, the GPMT test emerges as superior to the other two. All five guinea-pig techniques produced comparable false positive results (in relation to the human test results). There was no significant difference between any of the five guinea-pig test methods in this regard. False negative findings, meaning that the guinea-pig test failed to indicate an allergenic potential that was seen in human predictive testing, showed the highest frequency with the Draize and Buehler techniques and the lowest frequency with the GPMT.

The overall findings of these tests support the conclusion that the GPMT is the method of choice to best duplicate human predictive findings, as measured by the human modified Draize method.

Additional guinea-pig techniques

The standard Draize guinea-pig test and GPMT were used as references for evaluating the usefulness of a non-occlusive topical test and its possible enhancement with DMSO. In addition, we altered the Draize guinea-pig test (altered Draize) to find out whether a higher induction concentration would improve the Draize guinea-pig test, as it does the Draize human test.

The guinea-pig test results are shown in Table 4 and comparisons with human findings are shown in Table 5. The results here are not as dramatic as in the first series of tests, largely because there were fewer compounds tested (five) and fewer replicates (two). These results nevertheless repeat the earlier findings that the GPMT is the test of choice among the guinea-pig test methods evaluated. DMSO pretreatment of the induction site prior to application of a putative sensitizer did not make that site more prone

Table 3. *Validation of guinea-pig predictive test methods by comparison of results with those obtained by a human predictive test method*‡

Guinea-pig test method	Results (frequency/30 evaluations) compared with predictive test method in humans		
	Agreement	False positive	False negative
Draize	14	1	15
Buehler	10††	0	20††
Cy/CFA	23**†	0	7*†
GPMT	29**	0	1**
Split-adjuvant	22*†	2	6**†

Cy/CFA = Cyclophosphamide/complete Freund's adjuvant bioassay

GPMT = Guinea-pig maximization test

‡The results of the guinea-pig predictive tests are shown in Table 1. The results for DMSO have not been included for the comparison since it was not tested in humans. Three rounds of each test were carried out on each of the ten remaining chemicals (i.e. a total of 30 evaluations/test). Ten guinea-pigs were used for each evaluation. The results of the modified Draize test on humans are shown in Table 2. If one or more of the human subjects showed a reaction, the chemical was considered to be allergenic. If one or more of the guinea-pigs showed a reaction, an evaluation was considered to have shown a positive response.

Values marked with asterisks differ significantly (chi-square test) from those for the guinea-pig Draize test (*$P < 0.05$; **$P < 0.01$). Values marked with daggers differ significantly (chi-square test) from those of the GPMT (†$P < 0.05$; ††$P < 0.01$).

Table 4. *Results of two rounds of testing of five compounds using five guinea-pig bioassays for allergenicity*

Compound	Concn and vehicle†	Test	No. of guinea-pigs with reaction*		
			Round 1	Round 2	Cumulative
Hydroxycitronellal	20% (v/w) in petrolatum	Draize	0	0	0
		Altered Draize	0	0	0
		NTT	0	0	0
		NTT–DMSO	0	0	0
		GPMT	2	4	6
Methyl crotonate	6% (v/w) in petrolatum	Draize	0	0	0
		Altered Draize	0	0	0
		NTT	0	0(9)	0(19)
		NTT–DMSO	0	0	0
		GPMT	0	0(9)	0(19)
Vetivert acetate	20% (w/w) in petrolatum	Draize	1	0	1
		Altered Draize	2	0	2
		NTT	1	0	1
		NTT–DMSO	0	1	1
		GPMT	1	0	1
Dimethyl citraconate	12% (v/w) in petrolatum	Draize	0	0	0
		Altered Draize	0	0(9)	0(19)
		NTT	0	0(9)	0(19)
		NTT–DMSO	0	0(9)	0(19)
		GPMT	2	2(9)	4(19)
Germall 115	10% (w/w) in petrolatum	Draize	0	0	0
		Altered Draize	1	2	3
		NTT	5	5	10
		NTT–DMSO	3	3	6
		GPMT	4	4(9)	8(19)

NTT = Non-occlusive topical test NTT–DMSO = Non-occlusive topical test using DMSO
GPMT = Guinea-pig maximization test

*Ten guinea-pigs were used in each round (cumulative number = 20) except where the numbers used are indicated in brackets.

†For tests other than the Draize and altered Draize tests; a concentration of 0·1% test compound was used for the Draize test and in the altered Draize test a concentration of 0·5% was used.

to the development of contact sensitivity. Increasing the induction and challenge concentration of an allergen in the altered Draize test resulted in the identification of vetivert acetate as an allergen; this would have been missed in the standard guinea-pig Draize test. Further alteration of the guinea-pig Draize test along these lines might be worthwhile.

DISCUSSION

New cosmetics, new drugs and other substances that come into contact with human skin, and the allergenicity of which is unknown, are continually being developed. As part of an evaluation of their safety, it is necessary to measure their relative allergenic potential for contact dermatitis.

While allergic contact dermatitis can be induced in a variety of laboratory animals, such as mice, rats, hamsters and chickens, the guinea-pig remains the animal of choice for the bioassay of putative contact allergens of man (Asherson & Ptak, 1969; Jaffee & Maguire, 1981; Maguire, 1980; Maguire, Rank & Weidanz, 1976). Compounds that produce a high incidence of contact sensitivity in the human, i.e. strong and moderate sensitizers, are readily identified in the guinea-pig by classical techniques such as the guinea-pig Draize method (Draize, 1965) or the more recently developed Buehler method (Buehler & Griffith, 1975; Ritz & Buehler, 1980). However, in the case of weak and very weak contact sensitizers, these methods are less successful: more reliable methods for the evaluation of such compounds in the guinea-pig are needed (Magnusson & Kligman, 1977). While the incidence of sensitization with such compounds may be small, the population at risk often is very large so that significant numbers of individuals may be injured. For different compounds, the sensitization rates vary, the acceptable risk in a large part being determined by the conditions of use and the purpose of the final product.

Clearly, the testing of allergens in laboratory animals should be preliminary to their testing in humans, and the animal methods should be of such sensitivity and reliability as to preclude the exposure of large numbers of human volunteers to significant sensitizers. In product development, there is a distinct economic advantage to be derived from being able to exclude allergenic materials at a relatively early stage of development. In specific instances, it may be possible to reduce the allergenicity of chemicals of particular usefulness (chemical modification, change in vehicle). For this purpose, the need for sensitive, reliable bioassays of allergenicity that can readily be done in laboratory animals is obvious. Such testing in humans is inappropriate.

The efficiency of adjuvant techniques is clearly demonstrated in the present study. Suggestions of this

Table 5. *Comparison of results of sensitization tests in guinea-pigs with those of a predictive test method in humans*‡

Guinea-pig test method	Results (frequency/10 evaluations) compared with predictive test in humans		
	Agreement	False positive	False negative
Draize	3	0	7
Altered Draize	5†	0	5†
NTT	5†	0	5†
NTT–DMSO	5†	0	5†
GPMT	9*	0	1*

NTT = Non-occlusive topical test
NTT-DMSO = Non-occlusive topical test using DMSO
GPMT = Guinea-pig maximization test

†The results of the guinea-pig predictive tests are shown in Table 4. Two rounds of each test were carried out on each of five chemicals (i.e. a total of 10 evaluations/test). Ten guinea-pigs were used for each evaluation. The results of the modified Draize test on humans are shown in Table 2. If one or more of the human subjects showed a reaction the chemical was considered to be allergenic. If one or more of the guinea-pigs showed a reaction, an evaluation was considered to have shown a positive response.

Values marked with an asterisk differ significantly (chi-square test) from those for the guinea-pig Draize test ($*P < 0.01$). Values marked with a dagger are nearly significantly different (chi-square test) from those of the GPMT ($\dagger P > 0.05$ but < 0.1).

efficacy have been alluded to in other investigations as well (Magnusson & Kligman, 1969; Maurer, Thomann, Weirich & Hess, 1975). In addition, the present work demonstrates the overall superiority of the GPMT over two other candidate guinea-pig test methods with regard to its capacity to duplicate human predictive findings.

Finally, the results of this study provide a basis for further recommending that compounds about which little is known be tested first by the Draize guinea-pig technique and then by the GPMT. Positive findings in the Draize guinea-pig test would suggest that the compound is a strong skin sensitizer and may imply that further testing with the GPMT is unnecessary. Negative findings in both the Draize and the GPMT indicate that the chemical is not likely to be a significant sensitizer in humans. Positive findings in the GPMT with negative results in the Draize guinea-pig test suggest that the compound is likely to be a weak or moderate skin sensitizer in humans.

As part of this study the contact allergenicity of ethanol was evaluated. By the modified human Draize test none of the 94 subjects were sensitized (Table 2). Furthermore, we failed to sensitize any of 150 guinea-pigs to this substance (Table 1). Thus, a reported allergenicity of ethanol in humans (Stotts & Ely, 1977) was not confirmed.

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SHORT PAPERS

TERATOGENIC POTENTIAL OF QUERCETIN IN THE RAT

C. C. WILLHITE

Toxicology Research Unit, Western Regional Research Center, United States Department of Agriculture 800 Buchanan Street, Berkeley, CA 94710, USA*

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Abstract—A single oral dose of 2, 20, 200, or 2000 mg quercetin/kg was admin stered to pregnant rats on the morning of day 9 of gestation. Other groups of pregnant rats received similar oral doses of quercetin daily, on days 6–15 of gestation. Some quercetin-treated groups showed a significant decrease in the average weight of day-20 foetuses compared with the corresponding control weight. However, studies of the foetuses recovered on day 20 of gestation failed to reveal any reproducible dose-related syndrome of teratogenic effects attributable to quercetin treatment.

Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a common flavonol which occurs in both free and conjugated forms in a variety of edible plant products (Herrmann, 1976; Pamukcu & Bryan, 1979; Seino, Nagao, Yahagi *et al.* 1978; Wang, Chiu, Pamukcu & Bryan, 1976). Quercetin has been shown to be a frameshift mutagen in *Salmonella typhimurium* and although the flavonol showed some mutagenic activity without metabolic activation, its mutagenicity was enhanced in the presence of rat hepatic microsomal preparations (Bjeldanes & Chang, 1977; MacGregor & Jurd, 1978; Sugimura, Nagao, Matsushima *et al.* 1977). MacGregor (1979) found that rat urine contained substances mutagenic for *S. tryphimurium* following an oral or ip dose of quercetin. Others have provided data on the mutagenicity of quercetin in cultured mammalian cells (Maruta, Enaka & Umeda, 1979) and on the *in vitro* transformation of embryonic cells in the presence of quercetin (Umezawa, Matsushima, Sugimura *et al.* 1977). Pamukcu, Yalciner, Hatcher & Bryan (1980) demonstrated that chronic exposure of rats to 0·1% quercetin in the diet was carcinogenic for the intestinal and bladder epithelium.

Since many chemicals that have been linked to mutagenic and/or carcinogenic effects have also been shown to possess teratogenic activity (Kalter, 1971), it was of interest to evaluate quercetin for pathological effects on the developing rat embryo. The results of an assessment of the teratogenic potential of quercetin in the rat are presented and discussed in this paper.

*Mention of trade names, proprietary products or specific equipment does not constitute a guarantee or warranty by the United States Department of Agriculture, nor does it imply approval to the exclusion of other products that may be suitable.

Experimental

Timed pregnant Sprague–Dawley CD rats (body weight 200–240 g) were purchased from the Charles River Breeding Laboratories, North Wilmington, MA. The day following the evening of breeding was considered to be day 1 of gestation. The rats were maintained at 23°C and were fed laboratory chow and tap water *ad lib.* Pine shavings were used for bedding.

Quercetin (Sigma Chemical Co., St Louis, MO) was suspended in corn oil and administered by intubation in a dosage volume of approximately 1·0 ml/100 g body weight. The animals were treated either with a single dose of 2·0, 20·0, 200·0 or 2000·0 mg quercetin/kg on the morning of day 9 of gestation or with similar intubated doses on the mornings of days 6–15 of gestation. Appropriate groups of pregnant rats given an equivalent amount of corn oil alone served as controls. Reference teratogens were given to additional groups of pregnant rats of identical gestational age. A single oral dose of 200,000 IU vitamin A acetate (from Nutritional Biochemicals Corp., Cleveland, OH) made up in distilled, deionized water was given to each rat of one positive control group on the morning of day 9 of gestation. An additional group received a single sc injection of 160 mg trypan blue/kg (from Allied Chemical Corp., New York) made up in distilled, deionized water on the morning of day 9 of gestation.

The pregnant rats were killed with excess CO_2 on day 20 of gestation and the offspring were removed by caesarian section. The numbers of living foetuses and the numbers of resorption sites were recorded. The foetuses were examined under a binocular dissecting microscope for gross developmental malformations, and rib anomalies were studied through the translucent skin prior to fixation. The foetuses were then dried on absorbent paper, weighed, and either placed into Bouin's fluid and dissected after the method of Wilson (1965), or fixed in 95% ethanol and eviscer-

Table 1. *Prenatal effects of quercetin following single or repeated oral administration to rats on day 9 or days 6–15 of gestation*

Parameter	Data for groups given quercetin in dose(s) (mg/kg) of:				
	0†	2	20	200	2000
			Single dose on gestation day 9		
No. of treated dams	9	11	11	11	12
No. of litters	9	8	10	8	12
No. of implantation sites	90	81	94	79	116
Resorptions—no.	0	1	1	1	1
—%	0	1·2	1·1	1·2	0·8
No. of live foetuses/litter‡	10·0 ± 1·7	10·0 ± 2·3	9·4 ± 4·3	9·8 ± 2·6	9·6 ± 2·3
Foetal weight (g)‡	2·86 ± 0·39	2·75 ± 0·43	2·86 ± 0·32	2·72 ± 0·20*	2·72 ± 0·40*
			Daily dose on gestation days 6–15		
No. of treated dams	8	12	9	8	8
No. of litters	6	11	5	6	6
No. of implantation sites	58	113	40	60	66
Resorptions—no.	0	2	0	0	1
—%	0	1·8	0	0	1·5
No. of live foetuses/litter‡	9·6 ± 2·1	10·2 ± 2·2	8·0 ± 3·9	10·0 ± 2·4	11·0 ± 1·9
Foetal weight (g)‡	2·89 ± 0·38	2·57 ± 0·28*	2·94 ± 0·71	2·80 ± 0·54	2·41 ± 0·37*

† Control group given the vehicle (2 ml corn oil) only.
‡Values are means ± 1 SD; those marked with an asterisk differ significantly ($P < 0·05$ by Student's t-test) from the corresponding control value.

ated, in preparation for alizarin-red staining of the skeletons. The foetal body weights were analysed statistically using the unpaired Student's t test (Snedecor & Cochran, 1967). Values were considered to be significantly different at the 95% confidence interval.

Results

Quercetin treatment

No overt signs of intoxication were observed in dams from any of the experimental groups. Following treatment with very high doses of quercetin, a large proportion of the dose appeared to pass through the gastro-intestinal tract of the rat unchanged and to be excreted in the faeces. Yellow-coloured faeces were noted in the cages of quercetin-treated animals and these were most evident in those housing dams given the highest dose of quercetin. The mean number of living foetuses per litter in all quercetin-treated groups was similar to the control figure (Table 1). Litters taken from quercetin-treated mothers showed a low resorption rate and this rate was not substantially different from that of the corresponding control group. The mean weight of foetuses taken from dams treated with either 200 or 2000 mg quercetin/kg on the morning of day 9 of gestation was significantly less than the mean control weight, but this decrease in body weight was not evident in litters taken from mothers treated with either 2 or 20 mg/kg. The mean body weight of foetuses taken from dams treated with 2 or 2000 mg quercetin/kg on days 6–15 of gestation was significantly less than that of the control, but these differences were not evident at intermediate doses.

Gross examination of offspring taken from dams given corn oil alone on the morning of day 9 showed 89 animals to be normal, but one foetus was afflicted with exencephaly, umbilical hernia, microphthalmia and mandibular hypoplasia. The littermates of the affected control animal were grossly normal, as were all animals taken from dams treated with corn oil alone on days 6–15 of gestation; thus, this specimen can be considered to be a case of severe spontaneous congenital malformation. Two animals taken from one mother given 2 mg quercetin/kg and one foetus recovered from a dam treated with 20 mg/kg on the morning of day 9 had wavy ribs. Alizarin-stained preparations confirmed fusions of the ribs in two animals taken from a litter treated with 2000 mg quercetin/kg on days 6–15 of gestation. Dissection of foetal rats revealed unilateral testicular agenesis in two animals from one litter treated with 2 mg/kg on days 6–15 of gestation; it should be noted that one foetus from a dam treated with corn oil alone was similarly affected. In addition, one foetus from a dam treated with 200 mg quercetin/kg throughout organogenesis showed unilateral renal hypoplasia.

Positive controls

A single sc injection of 160 mg trypan blue/kg on the morning of day 9 of gestation was associated with a wide range of abnormalities including cranioschisis aperta with exencephaly, cranioschisis occulta with encephalocoele, gastroschisis, extensive internal hydrocephalus, unilateral renal agenesis and tail abnormalities. The resorption rate associated with this dose of trypan blue, a well-established rodent teratogen, was 26%. The increased resorption rate was very probably an indication that many of the more severely malformed embryos died *in utero*. Induction of hypervitaminosis A in the pregnant Sprague–Dawley Charles River CD rat has previously been described (Nolen, 1969). The most common defect induced by the vitamin in the present experiments was cranioschisis occulta with encephalocoele. These data indicate that this strain of rat was susceptible to teratogenic insult from a variety of chemicals and that the days selected for treatment were appropriate for

tests to detect possible teratogenic activity of the flavonol.

Discussion

Ambrose, Robbins & DeEds (1952) reported a low toxicity for quercetin in rats and rabbits exposed to the flavonol for up to 410 days. Petrakis, Kallianos, Wender & Shetlar (1959) studied the distribution and fate of quercetin in the rat. Approximately 80% of the radioactivity associated with an oral dose of 5 mg randomly labelled [^{14}C]quercetin was recovered in the 12 hr after dosing and, of that amount, over half was found in the intestinal contents. Most of the radioactivity was located in the lower bowel. Of the absorbed radioactivity, the lungs (with 12% of the dose) and the wall of the large intestine (with 3%) contained the greatest amount. The blood, kidneys and gastric walls accounted for $<1\%$ of the total dose, whereas no activity was detected in the liver, spleen, heart or brain. The urinary excretion accounted for 4% of the dose and expired CO_2 accounted for 15%. No radioactive quercetin *per se* was detected in the urine. Data obtained from other experiments with rats (MacGregor, 1979; Petrakis *et al.* 1959) have indicated that absorption and urinary excretion of approximately 0·3% of the parent flavonol and/or its mutagenic metabolites occurred following a single oral dose.

Gugler, Leschik & Dengler (1975) studied the pharmacokinetics of quercetin in man following a single iv or oral dose. The plasma data following iv injection of 100 mg quercetin were analysed according to a two-compartment model with half-lives of 8·8 ± 1·2 min (0–40 min) and 2·4 ± 0·2 hr (40–540 min). The apparent volume of distribution was 0·34 ± 0·03 litre/kg; protein binding in the plasma was estimated to be in excess of 98%. Only 0·65% of the iv dose was excreted unchanged and 7·4 ± 1·2% was excreted in urine as conjugated metabolites. An oral dose of 4 g quercetin in human volunteers (50–65 mg/kg) failed to result in measurable plasma concentrations ($<0·1$ μg/ml) and neither quercetin nor conjugated quercetin was detected in the urine at any time. The faecal excretion of quercetin at up to 72 hr after dosing accounted for 53 ± 5% of the dose and only traces of quercetin appeared in the faeces after that time. Gugler *et al.* (1975) ascribed the disappearance of the remainder of the dose to microbial degradation in the lower digestive tract and concluded that $<1\%$ of the quercetin was absorbed intact from the digestive tract. There was little evidence that free quercetin persisted in either general or enterohepatic circulation following ingestion of the flavonol.

It has been estimated that the daily intake of flavonoids in the average American diet is approximately 1 g and that some 50 mg 'quercetin equivalents'/day represents a reasonable estimate of dietary exposure (Brown, 1980; Kühnau, 1976). The doses of quercetin used in the present study included some that were greatly in excess of the levels likely to be consumed by women of child-bearing age. The data presented here indicate that neither a single oral exposure during a critical stage of embryonic development nor multiple oral doses of quercetin given throughout organogenesis represented a teratogenic threat in the rat.

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INHIBITION OF NITROSATION OF AMINES BY THIOLS, ALCOHOLS AND CARBOHYDRATES

D. L. H. WILLIAMS and S. E. ALDRED

Department of Chemistry, University Science Laboratories, South Road, Durham DH1 3LE, England

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Abstract—The addition of a number of simple alcohols and carbohydrates to reactions of nitrous acid with amines in dilute acid solution resulted in a reduction in the overall rate constant for *N*-nitrosation. The effects were not very large and complete suppression of nitrosation was not achieved. The results are all consistent with the rapid equilibrium formation of the corresponding alkyl nitrite which is virtually inactive as a direct nitrosating agent itself. Addition of the two thiols L-cysteine and *N*-acetylpenicillamine, however, had a much more marked effect and it was possible to prevent nitrosation of the amine completely in both cases. This finding was no doubt due to competition from the irreversible *S*-nitrosation. On the other hand the effect of *S*-methyl-L-cysteine and L-methionine was rather small. The results are all discussed in terms of the likely reaction mechanisms.

Introduction

Interest continues to be high in the area of the chemistry of nitrosamines, from the point of view of both their formation and their reactions, because of the potential risk to humans from carcinogenic nitrosamines formed *in vivo* from secondary (and tertiary) amines and nitrous acid. It is known that certain anions, notably Cl^-, Br^-, I^- and SCN^- catalyse the formation of nitrosamines in acid solution (Ridd, 1961), and the mechanisms of the reactions are reasonably well understood. Equilibrium concentrations of the corresponding nitrosyl halides (or thiocyanate) are formed, and these act as more powerful nitrosating agents than does nitrous acid itself. Conversely there are many reagents that inhibit nitrosation by reacting competitively with nitrous acid in an irreversible manner; these include added sodium azide, hydrazine, sulphamic acid, ascorbic acid, hydroxylamine and urea. A method has been developed (Williams, 1975) on the basis of a kinetic analysis which establishes the relative reactivities of these nitrous acid traps or scavengers. The above sequence represents the decreasing order of reactivity.

A more detailed comparison of the efficiencies of sulphamic acid and ascorbic acid has been presented (Williams, 1978) for reaction at different mineral acid concentrations and in the presence of bromide ion and thiocyanate ion. As part of an extension of this work with nitrous acid scavengers we have sought to examine the efficiencies of a range of alcohols and several thiols in this context. It is well-known (Smith, 1966) that rapid equilibrium conversion of nitrous acid to the corresponding alkyl nitrite occurs in the presence of alcohols. Recently the rate constants for the forward and reverse reactions have been measured by stopped-flow spectrophotometry (Aldred & Williams, 1980). It has also been demonstrated that cysteine undergoes rapid and essentially irreversible *S*-nitrosation (Collings, Al-Mallah & Stedman, 1975).

Experimental

The basic reactions studied were the nitrosation of *N*-methylaniline and *p*-nitro-*N*-methylaniline. Commercial samples of these two amines were purified by distillation and recrystallization, respectively. All other materials used were of the highest grade of purity available and were used without further purification.

Rate measurements were carried out in a recording UV-visible spectrophotometer, either the appearance of absorption at 275 nm due to the nitrosamine (for *N*-methylaniline) or a decrease in the absorption at 405 nm due to the amine (for *p*-nitro-*N*-methylaniline) being noted. All measurements were carried out at 31°C in aqueous solution with about a 20-fold excess in the concentration of sodium nitrite over that of the amine, and in the presence of dilute mineral acid. Under these conditions, good first-order behaviour was observed for at least two half-lives, and the first-order rate constant k_0, defined by $-d[\text{Amine}]/dt = k_0[\text{Amine}]$, was calculated in the usual way from the integrated rate equation.

Results and Discussion

Table 1 shows the variation in the first-order rate constant for the *N*-nitrosation of *N*-methylaniline in the presence of increasing concentrations of methanol. The rate constants decrease steadily as the methanol concentration is increased, resulting in an approximately tenfold reduction in k_0 for $[CH_3OH] = 0{\cdot}989$ M. In each case the yield of nitrosamine was the same (virtually quantitative). Similar results were found for the following alcohols—ethanol, 1-propanol, 2-propanol, ethanediol and 1,3-propanediol, although the reduction in k_0 for each of these alcohols was not quite as marked as that for methanol. Similarly the effect of added carbohydrates

Table 1. *Rate constants for the nitrosation of* N-*methylaniline (NMA)* in the presence of methanol*

Concn of methanol (M)	$10^3 k_0/s^{-1}$
0	43·6
0·247	20·3
0·494	11·7
0·642	7·9
0·742	6·9
0·989	4·8

*$[NaNO_2] = 6{\cdot}0 \times 10^{-3}$ M; $[H_2SO_4] = 0{\cdot}08$ M; $[NMA] = 1{\cdot}86 \times 10^{-4}$ M.

was investigated with mannitol, glucose, sucrose, maltose and lactose. The data for maltose, which are typical, are shown in Table 2. Again, in each case a marked decrease of k_0 was found as the carbohydrate concentration was increased.

The results for all of the alcohols and carbohydrates studied are consistent with a mechanism whereby the nitrous acid is partially and rapidly converted to the alkyl nitrite, leaving a lower concentration of available HNO_2 to effect the N-nitrosation (see Scheme 1).

$$HNO_2 + ROH \rightleftharpoons RONO + H_2O \text{ (fast equilibrium)}$$

$$HNO_2 + \text{Amine} \rightarrow \text{Nitrosamine} \quad \ldots\ (1)$$

This analysis depends on the alkyl nitrite's not being an active nitrosating agent itself. Recent work with *n*-propyl nitrite (Aldred & Williams, 1981) has shown that this is indeed the case. The usefulness of alkyl nitrites as nitrosating agents depends on their prior hydrolysis to nitrous acid or, if there are suitable nucleophiles present, on the formation of the reactive nitrosyl halide or other reactive derivative. A more detailed kinetic analysis (not presented here) enables values of K, the equilibrium constant for RONO formation, to be obtained from the data in Tables 1 and 2. In all cases the values agree quite well with those determined directly.

Table 2. *Rate constants for the nitrosation of* N-*methylaniline (NMA)* in the presence of maltose*

Concn of maltose (M)	$10^3 k_0/s^{-1}$
0	9·65
0·115	7·84
0·147	7·33
0·207	6·83
0·265	6·13

*$[NaNO_2] = 2{\cdot}0 \times 10^{-3}$ M; $[HClO_4] = 0{\cdot}04$ M; $[NMA] = 1{\cdot}2 \times 10^{-4}$ M.

It has been reported on a number of occasions (e.g. by Walker, Pignatelli & Castegnaro, 1975) that certain phenolic substances can *catalyse* the nitrosation of amines. We found no such effect for the alcohols studied here and preliminary studies with phenol itself have shown that it exerts little effect upon the rate constant for the nitrosation of *p*-nitro-*N*-methylaniline when added at concentrations around 2×10^{-3} M.

We have also examined the effect of the addition of two thiols, L-cysteine and *N*-acetylpenicillamine, upon *N*-nitrosation. For ease of spectral measurements we chose to examine the nitrosation of *p*-nitro-*N*-methylaniline and to follow the disappearance of the absorption due to this amine at 405 nm. The corresponding nitrosamine was observed at 310 nm. As the concentrations of either cysteine or *N*-acetylpenicillamine were increased, the extent of nitrosation decreased markedly and it was possible to inhibit nitrosamine formation completely when the added thiol was in *c.* 20-fold excess over the amine. As expected, if the nitrous acid and thiol solutions were pre-mixed for around 45 sec before addition of the amine, much less nitrosamine was formed. It is likely that we are observing a direct competition between *S*- and *N*-nitrosation as outlined in scheme 2.

$$HNO_2 + RSH \rightarrow RS\overset{+}{\underset{|}{\underset{H}{}}}NO \rightarrow RSNO$$

$$HNO_2 + R'R''NH \rightarrow R'R''\overset{+}{\underset{|}{\underset{H}{}}}N{-}NO \rightarrow R'R''NNO \quad \ldots\ (2)$$

The essential difference between the thiol and alcohol systems appears to be not so much in the relative rates of *O*- and *S*-nitrosation, but in the virtual irreversibility of *S*-nitrosation compared with *O*-nitrosation. We have used here a very reactive amine; for reactions of the less reactive dialkylamines under these conditions, the inhibitory effect of added thiols should be even more marked.

The effect of addition of the disulphides L-methionine and *S*-methyl-L-cysteine to these reactants has also been examined in a preliminary way. Here, very large concentrations are required to eliminate *N*-nitrosation completely, and this was not achieved totally in the case of methionine. It was found for *S*-methylcysteine that a concentration excess over the nitrous acid of c.750 was necessary. Presumably this arises because the *S*-nitroso ion first formed cannot lose a proton as is the case with the thiols and hence the reaction is probably significantly reversible.

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IMMUNE STUDIES WITH T-2 TOXIN: EFFECT OF FEEDING AND WITHDRAWAL IN MONKEYS

V. JAGADEESAN, C. RUKMINI, MALINI VIJAYARAGHAVAN and P. G. TULPULE

National Institute of Nutrition, Indian Council of Medical Research, Jamai Osmania (P.O.), Hyderabad-500 007 (A.P.), India

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Abstract—Ingestion of T-2 toxin, a product of Fusarium fungi, has been reported to have a variety of effects leading to morbidity and mortality in animals and humans. Semi-purified T-2 toxin was given to monkeys by gastric intubation at a level of 100 μg/kg body weight/day for 4–5 wk and the haematological and immune parameters were studied before and after the treatment. Leucocyte counts were depressed at the end of wk 4 of treatment. The immunological studies showed suppression of the bactericidal activity of neutrophils, of cell-mediated immune status as assessed by T-cell number and lymphocyte transformation, and of humoral immunity as reflected in B-cell number and IgG and IgM levels. However serum complement (CH50) did not show any change. Investigations carried out 5 months after withdrawal of the toxin indicated that these parameters had returned almost to the initial, pretreatment levels. These data suggest that the greater incidence of infection seen in mycotoxin-ingesting animals may be due to immune suppression. Withdrawal of the mycotoxin results in improvement of haematological and immune functions.

Introduction

It is well known that mycotoxins have a number of important biological effects in animals and man (Rodricks, Hesseltine & Mehlman, 1977). In many situations, animals ingesting the toxin are rendered susceptible to infection, which may at times result in death. However, the mode of action of these fungal toxins in causing morbidity or mortality has received only scant attention. Among the mycotoxins, only aflatoxin has been investigated in detail, with studies that have included its effect on mechanisms of immunity and native resistance (Pier, Richard & Thurston, 1980). The relevance of immunology to toxicological evaluation has been stressed recently by Miller (1981).

Fusarium toxins (trichothecenes) have been implicated in cattle mycotoxicoses (Rodricks & Eppley, 1974), and alimentary toxic aleukia in man has been attributed to the consumption of Fusarium-infested cereal grains (Joffe, 1974). Isolation of the active principle in Fusarium mould, namely T-2 toxin, and its effects when fed to experimental animals were reported earlier from this Institute (Rukmini & Bhat, 1978; Rukmini, Prasad & Rao, 1980). A marked leucopenia was observed in monkeys consuming the toxin, and prolonged feeding resulted in haemorrhage and respiratory-tract infection, leading ultimately to death. The haematological and histological findings suggested that T-2 toxin might exert its action by suppressing the immunological mechanisms of the host. A study was therefore initiated to investigate the effects of the feeding of T-2 toxin on various aspects of immunity. In addition, effects on the immune changes, if any, following withdrawal of the toxin were also studied.

Abbreviations: PCV = Packed cell volume; PHA = Phytohaemagglutinin.

Experimental

Test material. Semi-purified T-2 toxin was prepared by the procedure described earlier (Rukmini & Bhat, 1978). In brief, *Fusarium 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. 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.

Animals and treatment. Seven male rhesus monkeys (body weight 2–3 kg and free from any apparent infection) were caged individually and fed a stock diet containing adequate amounts of all essential nutrients. Each monkey served as its own control. Fasting blood samples were collected initially for haematological and immunological studies. Subsequently, all animals were given a daily dose of semi-purified T-2 toxin (100 μg/kg body weight/day) in milk by stomach tube for a period of 4–5 wk. At an early stage of treatment, three animals developed complications, notably vomiting, haemorrhage and respiratory infection, and died. In the other four animals, the leucocyte count in the blood had fallen by more than 40% of the initial count by the end of wk 4, at which time the immune studies were repeated. Administration of the toxin was then stopped and the studies were carried out again after a lapse of a further 5 months.

Table 1. *Haematological studies in monkeys given 100 μg T-2 toxin/kg/day by gastric intubation for 4–5 wk*

Sampling time	Hb (g/dl)	PCV (%)	RBC ($\times 10^6$/mm^3)	Total WBC ($\times 10^6$/ml)
Pretreatment	15·2 ± 0·51	44 ± 1·4	5·2 ± 0·54	12·4 ± 0·88
End of treatment	14·1 ± 0·26	42 ± 1·1	5·5 ± 0·34	7·3 ± 0·49
After withdrawal*	14·2 ± 0·56	43 ± 1·4	5·6 ± 1·9	10·8 ± 0·67

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
WBC = White blood cells
*Samples were taken 5 months after the termination of treatment.
All values are means ± SEM for four animals.

Haematological and immunological methods

Haematology. Haemoglobin and PCV determinations and erythrocyte and total leucocyte counts were carried out by conventional methods.

Immunology. Several parameters were studied to assess the functional capacity of the various immune systems.

(i) Bactericidal capacity of leucocytes was measured by the method described by Selvaraj & Bhat (1972). Polymorphonuclear neutrophils in whole blood were incubated for 60 min with *Escherichia coli* in a 1:2 ratio in a medium containing 20% autologous plasma. At the end of the incubation period, serial dilutions were made and viable bacteria were plated on nutrient agar plates. Results were expressed as the $\log_{10}$ reduction in viable *E. coli* count at the end of the 60-min incubation. This test measured the ability of the neutrophils to engulf and kill the bacteria.

(ii) Cell-mediated immunity was assessed from T-lymphocyte counts and measurement of the response of these cells to PHA-induced blastogenesis.

T-lymphocyte counts were carried out by the rosette-formation technique. About 4 ml heparinized blood was thoroughly mixed with 10 ml 3% gelatin in saline and allowed to stand for 30 min at 37 C. The supernatant, rich in lymphocytes, was pipetted out and gently centrifuged to harvest the cells. The conventional E-rosette test for the detection of T lymphocytes was then carried out. The T-cell count was expressed as a percentage of the total leucocyte count.

To assess T-lymphocyte function by measuring the response of the cells to PHA-induced blastogenesis, peripheral-blood cultures containing a known number of lymphocytes were stimulated with 0·1 ml PHA (Burroughs Wellcome, Beckenham, UK). Control cultures were set up without PHA. At the end of 48 hr, 1 μCi [^{3}H]thymidine was added to each culture tube and the incubations were continued for another 24 hr. At the end of this period, the cells were spun, ^{3}H-labelled DNA was isolated and the radioactivity was counted in a liquid scintillation spectrometer. The results were expressed as the T/C ratio, i.e. (counts/min in test culture)/(counts/min in control culture).

(iii) Humoral immunity was assessed from B-cell counts and estimations of immunoglobulins and serum complement.

The conventional EAC-rosette technique was used to estimate the number of B cells, after initial separation of lymphocytes from the peripheral blood. The results were expressed as the percentage of B lymphocytes in the total leucocyte count.

The immunoglobulins, IgG and IgM, were estimated by the radial immunodiffusion method of Mancini, Carbonara & Heremans (1965) and serum haemolytic complement (CH50) was estimated by the method of Kabat & Mayer (1961).

Results

Haemoglobin and PCV determinations and erythrocyte counts showed no change after toxin administration (Table 1). The total leucocyte count was markedly reduced from an initial mean value of 12·4 × 10^6/ml to 7·3 × 10^6/ml. However, this effect showed some reversal (to 10·8 × 10^6/ml) after withdrawal of the toxin.

The results of the immunological studies are presented in Table 2. The bactericidal activity of leu-

Table 2. *Effects of gastric administration of 100 μg T-2 toxin/kg/day for 4–5 wk on various immune parameters in the monkey*

Sampling time	Bactericidal assay*	T-cell count (%†)	T/C ratio‡	B-cell count (%†)	Immunoglobulin levels (mg/dl plasma) IgG	IgM	Complement (CH50 units/ml serum)
Pretreatment	2·2 ± 0·47	32 ± 6·2	35·2 ± 7·94	20 ± 5·9	1285 ± 143·5	120·7 ± 13·42	65·2 ± 5·10
End of treatment	1·0 ± 0·18	18 ± 2·4	20·8 ± 5·44	11 ± 1·0	485 ± 53·4	100·3 ± 11·02	56·7 ± 8·00
After withdrawal§	1·8 ± 0·29	27 ± 2·6	31·7 ± 8·27	18 ± 0·9	876 ± 112·1	100·6 ± 5·66	59·6 ± 3·86

*Expressed as the $\log_{10}$ reduction in the viable count of *Escherichia coli*.
†Percentage of total leucocytes.
‡Lymphocyte transformation by PHA expressed as (counts/min in test culture)/(counts/min in controls).
§Samples were taken 5 months after the termination of treatment.
All values are means ± SEM for four animals.

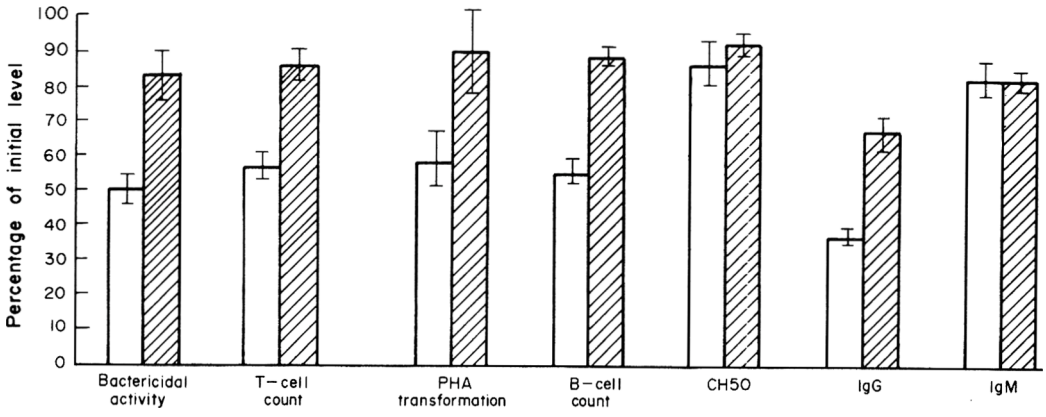

Fig. 1. Immune suppression in monkeys treated gastrically with 100 μg T-2 toxin/kg/day for 4–5 wk. Immune parameters determined at the end of the treatment period (□) and 5 months after termination of treatment (▨) are expressed as the mean percentage (with SEM) of the initial (pretreatment) level for four monkeys.

cocytes, T- and B-cell numbers, lymphocyte transformation by PHA, and immunoglobulin levels were all depressed by the treatment, but although the effects were marked, the changes were not statistically significant (paired *t* test) because of wide individual variation. However, all four animals given the toxin showed the reduction in neutrophil function, and in the other parameters, three of the four animals demonstrated the suppressive effect of the toxin. Serum complement levels did not show any appreciable fall. Five months after withdrawal of the toxin, the affected parameters were almost restored to the pretreatment levels. Only the mean IgM level showed little reversal, but the depression of this parameter during treatment was also less marked (about 16% of the initial level) than the fall in the other measurements. The percentage depression of each parameter as a result of toxin administration is represented in Fig. 1.

Of the three animals that died in the earlier stage of the experiment, only one could be autopsied. This animal received the toxin for 5 days before developing complications. Various organs were dissected out immediately after death and histopathological studies were carried out. There was evidence of acute fibrous pericarditis, and pneumonitis with abscesses was seen in both lungs. Lymph nodes showed cortical lymphocyte depletion, but the kidneys, liver, spleen and adrenals showed no change.

Discussion

It is known that when conditions favour their growth, Fusarium fungi can infest various cereal grains, including wheat, barley and sorghum. Fusarium has been implicated as the causative agent in mouldy-grain toxicosis such as corn disease in cows and horses and alimentary toxic aleukia in man (Joffe, 1974; Smalley & Strong, 1974). However, there is not much information on the mechanism of action of the toxin. Recently, the toxicological evaluation of the mycotoxins T-2 and T-2 tetraol has been attempted, using normal human fibroblasts *in vitro* (Oldham, Allred, Milo *et al.* 1980).

In the present study, the considerable fall observed in the total leucocyte count within the 4–5-wk treatment period confirms our earlier report (Rukmini *et al.* 1980).

The killing of bacteria by neutrophils and macrophages is thought to be the first line of defence in the body's immune mechanism. Earlier workers have shown impairment of phagocytosis due to ingestion of mycotoxins in different species of animal (Michael, Thaxton & Hamilton, 1973; Richard & Thurston, 1975). The present study showed that neutrophil function was impaired by gastric administration of T-2 toxin in all four animals studied. Recently Pokrovsky, Kravchenko & Tutelyan (1972) have demonstrated that aflatoxin ingestion can have a labilising effect on lysosomal membranes, causing the release of lysosomal enzymes. It is possible that such a mechanism may also operate in animals fed T-2 toxin, resulting in neutrophil dysfunction.

Cell-mediated immunity is one of the key defence mechanisms of the body and is mediated by the T-lymphocytes. T-cell numbers and *in vitro* stimulation by PHA were both depressed after the T-2 treatment but returned to near-normal levels after withdrawal of the toxin. Similar results indicating that the toxin interfered with the normal functioning of lymphocytes were obtained with regard to humoral immunity also, the B-cell number and serum immunoglobulin levels being reduced. Such an effect has already been demonstrated in aflatoxin-fed animals, and aflatoxin added at different levels to human lymphocytes in culture inhibited PHA-induced blastogenesis (Savel, Forsyth, Schaffer & Cardella, 1970). In guinea-pigs, aflatoxin depressed delayed cutaneous hypersensitivity and lymphokine activity (Pier, Fichtner & Cysewski, 1976). Tung, Wyatt, Thaxton & Hamilton (1975) and Giambrone, Ewert, Wyatt & Edison (1978) reported a reduction of IgG and IgM levels in different species of animals as a result of the feeding of aflatoxin—an effect similar to that observed in the present study after oral administration of T-2 toxin to

monkeys. Whether these toxins, aflatoxin and T-2 toxin, act at the bone-marrow level or interfere with the normal functioning of the circulating lymphocytes is not known. The differential count of the peripheral blood smear was suggestive of lymphocytopenia and the number of rosetting lymphocytes was also reduced at the end of the experimental period. Thus it seems likely that reduction in the number of lymphocytes may be the primary cause of the impaired immunity, although interference in the effector side of lymphocyte function cannot be ruled out. Neither is it known whether receptors for T-2 toxin exist in the lymphocytes, by analogy with zearalenone which has uterine receptors (Tashiro, Kawabata, Naoi & Ueno, 1980).

Serum complement is a group of circulatory proteins, the role of which as a non-specific immune mediator is becoming increasingly recognized (Whicher, 1978). Apart from the resolution of antigen–antibody complexes, its action in direct bacterial and viral killing and as a mediator of enhanced phagocytic action has been demonstrated. Studies by Thurston, Richard, Cysewski *et al.* (1972) and Richard, Thurston, Deyoe & Booth (1974) indicated that guinea-pigs given either aflatoxin or rubratoxin showed a significant mean depression in complement titres. In the present study no such depression was observed. It suggests either that T-2 toxin does not inhibit the synthesis and activation of the complement components or that the level of toxin administered in this study (100 μg/kg body weight/day) was insufficient to bring about a change. Alternatively, it is possible that only individual components are diminished, as is the case with aflatoxin, which depresses only the level of C4. This aspect requires further study.

The overall effect of such immune depression probably renders the toxin-ingesting animal more susceptible to infection. However, this can be tested only by introducing infection directly into animals ingesting the mycotoxin. The results of our study indicate that the immune suppression is a transient phenomenon since all the depressive effects could be largely reversed in 5 months. If this is true in the human situation also, it may be considered to indicate that ingestion of infested grains may not leave a permanent effect on the immune system of the host, if exposure to the offending grains is short. To the best of our knowledge, this is the first investigation in which the immunological studies have been carried out not only during the oral administration of the toxin, but also some time after its withdrawal. Our conclusions, however, are based on observations in a limited number of animals only. The results presented here need to be confirmed in a larger number of animals. A study of this type in experimental animals may be useful for evaluating safety limits for the intake of various mycotoxins.

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EFFECT OF A PROPRIETARY RUBEFACIENT "TIGER BALM"® ON RABBIT SKIN

L. GUPPY*, N. R. LOWEST and M. J. A. WALKER*

*Department of Pharmacology, Faculty of Medicine, The University of British Columbia, Vancouver, BC, V6T 1W5
and
†Western Veterinary Diagnostic Laboratories, Vancouver, BC, Canada

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Abstract—The effect of continuous exposure to proprietary rubefacients (Tiger Balm Red and Tiger Balm White), which contain menthol and camphor as well as clove, cajuput and cassia oils, was tested in rabbits. Dermal irritancy was determined by a 21-day continuous-application patch test (0·5 g/patch) and was assessed according to the Draize method of scoring. At the end of the 21-day test period, the skin was also examined histopathologically. In these tests, Tiger Balm Red (which contained 5% cassia oil plus 5% clove oil) caused irritation consisting of erythema, eschar formation and some oedema, to which a degree of tolerance developed. This irritation resulted in hyperkeratosis and sometimes inflammatory changes but no major damage to the skin. Tiger Balm White (no cassia oil and 2% clove oil) was better tolerated and produced less irritation and histological change than either Tiger Balm Red or a mixture of commercial waxes similar in composition to the wax base for Tiger Balm Red. None of the treatments produced any signs of systemic toxicity.

Introduction

Tiger Balm is a rubefacient formulated according to standard pharmaceutical practice in two forms, Red and White. The main ingredients are camphor, menthol, clove oil and cajuput oil (BP or BPC; see Table 1). Tiger Balm, in various forms, has been used for years as an externally applied ointment for the relief of muscle aches and sprains. The product is available for use in Asia, parts of Europe, the USA and Canada.

There is little clinical or experimental information regarding the skin irritancy of Tiger Balm and so we performed standard dermal tests in white rabbits to assess irritancy. As a predictor for potential irritancy in human skin, tests on rabbits are generally very satisfactory (Kligman & Wooding, 1967). Tests were therefore performed in the standard manner for a 21-day continuous application patch test (Steinberg, Akers, Weeks *et al.* 1975) at the end of which the animals were killed and examined for gross pathological abnormalities, while skin samples were subjected to standard histopathological examination.

Experimental

Test material. Tiger Balms Red and White (Lot numbers 80 A 06 and 79 E 02, respectively) were dispensed in standard 19·4-g commercial jars, obtained from the manufacturer in Singapore. Material was stored at 4°C and only sufficient containers for 7 days' application were allocated and kept at room temperature during use. For application, samples (0·5 g) were taken with a suitably calibrated spatula.

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Test animals. The 21 male and 21 female rabbits used were New Zealand albinos and weighed 2·5–3·0 kg at the beginning of the experiment. Animals were caged individually and allowed food (Purina Rabbit Chow, from Ralston Purina Inc., St Louis, MO, plus supplemental carrots) and tap-water *ad lib.* Cages were cleaned daily. The experimental rooms were on a 12-hr light/dark cycle with a mean temperature of 23·0 ± 0·1°C and humidity of 60 ± 2%.

The animals were acclimatized for 3 wk prior to use, and close clipping of the treatment area was carried out at regular intervals with an Oster clipper for 2 wk prior to the first applications of Tiger Balm. Clipping was repeated as necessary (to remove hair over 4 mm in length) and, although care was taken to avoid damaging the skin during the clipping, the process caused some animals to develop light sub-dermal haemorrhages, which took a day or two to disappear.

Treatment. Tiger Balm Red, Tiger Balm White or a control wax (a 17:46, w/w, mixture of commercial hard and soft waxes) was applied for 21 consecutive days according to the following experimental design. Each animal had four clipped areas—to the right and left of the dorsal mid-line and above and below the abdominal mid-line. On a random basis, two areas were abraded, by cross-hatching with the side of a No. 12 needle, and two were left unabraded. Abrasion was repeated at 5-day intervals. Treatment was allocated randomly, but in such a way that each animal received only one treatment (Tiger Balm Red, Tiger Balm White or control wax). In each animal, one abraded and one unabraded area received treatment throughout the whole 21-day schedule. Each animal therefore had two treated areas (abraded and unabraded) and two control areas (abraded and unabraded). Equal numbers of animals (seven) received each type of treatment.

Table 1. *Composition of Tiger Balm Red and Tiger Balm White*

Components*	Levels (%, w/w)	
	Tiger Balm Red	Tiger Balm White
Menthol	10	8
Camphor	25	25
Clove Oil	5	2
Cajuput Oil	7	13
Hard Paraffin	17	26
White Paraffin	24†	11‡
Peppermint Oil	6	15
Cassia Oil	5	0
Strong ammonia solution	1	0

*All ingredients are to BP or BPC standards. Analysis by the Huntingdon Research Centre (1976).
†Yellow.
‡White.

Aliquots (0·5 g) of material were placed onto the centre of gauze squares and spread into a circle (3–4 mm in diameter) corresponding to a circle drawn on the skin. The gauze was taped down and covered with an elasticized bandage, which was wrapped around the abdomen in such a manner as to cover all the gauzes and yet allow the animal to breathe freely. Material was applied to the skin continuously for 23 hr. It was then removed and the skin was cleansed with sterile saline to remove any obvious debris. The cleaned animals were then returned to their cages for 30 min prior to the reading of the skin areas for irritancy according to the Draize score (MacMillan, Rafft & Elvers, 1975).

Wherever possible, the skin was read 'blind', the reader being unaware of the treatment or even of which areas were treated and which were control. However, in the case of Tiger Balm Red it was not always possible to remove all red colouration, thereby leaving a clue as to treatment. The Draize scoring system used allocated a 1–4 integer score for erythema and eschar formation (A) and a 1–4 integer for oedema (B). The total score equalled the *score for A* plus the *score for B* giving a maximum possible score of 8. For these criteria, a score of 0–2 was considered mild irritation, 2–5 moderate irritation and 6–8 severe irritation. When scoring the animal according to the Draize criteria, readers noted reactions not falling within the above classification.

After the scoring, animals were re-treated, treatment being continued for a total of 21 days. All animals were weighed daily and a careful note was taken of their health. At the end of the 21-day treatment period all animals were killed by stunning and exsanguination and were subjected to a general autopsy examination prior to the removal of skin samples for histopathological study (all of which was carried out by Western Veterinary Diagnostic Laboratories, Vancouver). A mid-line abdominal incision was used to expose the inner organs, which were all examined grossly and weighed. Representative and suspicious organs were examined histologically.

Skin-sample histopathology. The mid-torso portion of the pelt was removed and the areas underlying the application areas were closely examined for reactions or possible necrosis. The whole of the treatment area, plus a 1·0-cm boundary, was then excised and stretched gently onto cork boards with pins, prior to fixation in neutral buffered formalin. Following fixation, suitably coded skin samples were prepared for embedding in wax in a random order. The sample to be embedded was taken from the centre of the application area and included a cross-section of treated and untreated skin. Following sectioning, two sections were stained with haematoxylin and eosin and were examined conventionally under the light microscope. All examinations were made on coded samples before the code was broken. Pathological findings were noted qualitatively, but the sections were also coded 1 for normal skin, 2 for hyperkeratosis and 3 for hyperkeratosis with inflammatory changes.

Results

In all animals, treatment with either Tiger Balm Red, Tiger Balm White or control wax, produced some degree of skin irritation. The mean irritancy scores for each of the treatments on both abraded and unabraded skin in males and females are shown in Fig. 1, which illustrates changes in mean Draize scores with treatment time. To calculate the Draize score for each rabbit treatment, the score for the control area (no treatment, except for covering) was subtracted in each individual case from the score for the corresponding treated area. This calculation gave two readings for each rabbit (i.e. treated minus control for the abraded area and treated minus control for the unabraded area). In most cases the score for the control area was 0. The maximum control score ever encountered was 3, and this was seen only once in over 800 readings.

Representative data (those for days 7, 14 and 21 of treatment) were subjected to analysis of variance (Anova) to give statistical-significance comparisons. In an overall analysis, treatment was a significant source of variance at all treatment days in both abraded and unabraded skin. At day 7, sex was also a significant source of variance. In comparison with the

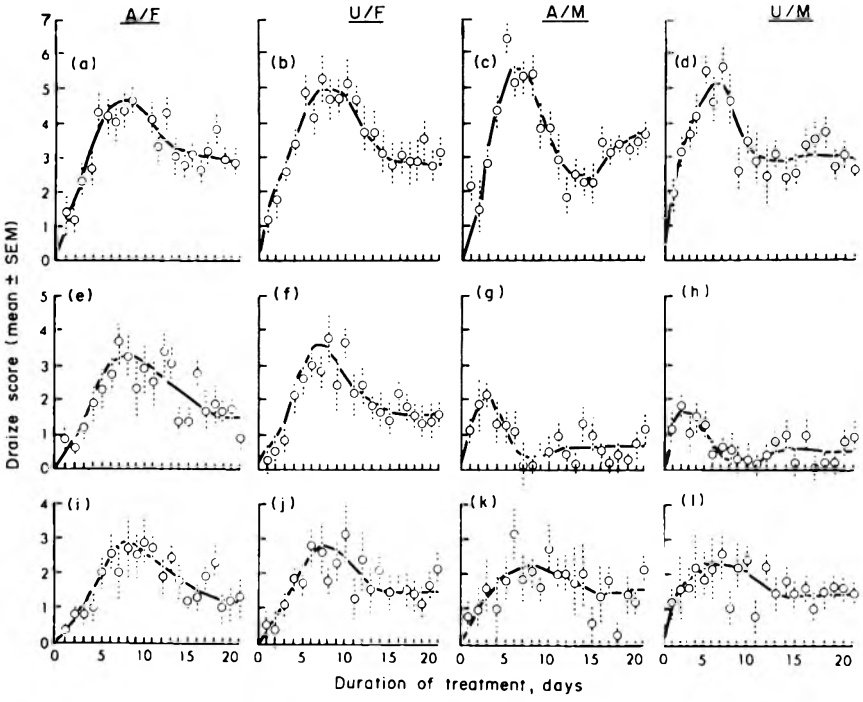

Fig. 1. Variations in Draize scores for abraded (A) and unabraded (U) rabbit skin during continuous treatment for 21 days with Tiger Balm Red (top row, graphs a–d), Tiger Balm White (e–h) or wax (i–l), as described in Experimental. Each point shows the mean score for a group of seven rabbits and the vertical rows of graphs present data for (from left to right) abraded and unabraded female (F) skin and abraded and unabraded male (M) skin.

scores for Tiger Balm Red, Tiger Balm White treatment often resulted in statistically lower scores, especially in males, while treatment with wax sometimes resulted in statistically lower scores. The lines shown in Fig. 1 are the suggested lines of best fit for the relationship between days of treatment and Draize score.

As can be seen from Fig. 1, all the treatments produced positive responses. These were most marked for Tiger Balm Red in both females (Fig. 1a,b) and males (Fig. 1c,d). In neither sex were there differences in the scores for abraded and unabraded skin. It is interesting to note that it took at least 7 days for responses to Tiger Balm Red to reach a maximum in all cases and that a tolerance developed with time, so that by the end of the 21-day treatment mean responses were 40% lower than the maximum responses. The degree of variation between the animals was relatively large, as is shown by the errors. The standard deviation, within a group, was some 20–50%, expressed as a percentage of the mean, indicating that, while one animal might score 7 for one treatment, another might score as little as 3. However, for any one animal, scores were consistent when a second blind reading was made on any day of treatment.

The scores for abraded and unabraded skin treated with Tiger Balm White (Fig. 1e–h) show, for female rabbits, a time-response pattern similar to that seen with Tiger Balm Red. Again, the response took at least 7 days to reach a maximum and thereafter tolerance developed. The maximum mean response obtained with Tiger Balm White on female skin was, however, lower than that seen with Tiger Balm Red, a peak response of about 5·0 being seen with Red, compared with about 3·0 with White. The response of male rabbits to Tiger Balm White (Fig. 1g,h) was much less vigorous than that in females and was con- or females (Fig. 1i, l); in both sexes the responses reached a peak of about 2·5 after 7 days. Statistical of day 7 of treatment with Tiger Balm White, male rabbits showed virtually no skin response. A mixture of waxes, similar in proportion to the waxes used in Tiger Balm Red, was not entirely innocuous in males or females (Fig. 1i,l); in both sexes the responses reached a peak of about 2·5 after 7 days. Statistical analysis of the data for treatment-days 7, 14 and 21 showed no differences in the responses to the wax between males and females or between abraded and unabraded areas.

These data were all collected before the rabbits were killed at the end of the 21-day treatment. During treatment none of the animals showed any signs of systemic toxicity or disease and at autopsy no gross abnormalities were found in any of the major organs or tissues in any of the rabbits. Where samples were taken and examined histologically, no abnormalities were found apart from signs of pneumonia in one rabbit.

The major organs were removed at autopsy and weighed. The results obtained by comparing weights uncorrected for body weight did not differ from those obtained when organ weights were expressed in terms of final body weights. Analysis of both sets of organ weights and the body weights showed that, except for

sex, only one statistically significant difference could be identified, namely that males treated with Tiger Balm Red had significantly heavier spleens.

The majority of samples taken from untreated, but covered, clipped skin were entirely normal, with no signs of pathological changes. In some cases, however, hyperkeratosis was observed, accompanied in less than 5% of the samples by a limited inflammatory response.

The treated skin samples showed an increased incidence of hyperkeratosis and inflammatory changes. The lowest level of such responses was seen in males treated with Tiger Balm White and the highest levels were seen in females and males treated with Tiger Balm Red (Table 2). Compared with untreated control skin, the major findings in skin treated with Tiger Balm White were a slightly increased incidence of hyperkeratosis and inflammatory changes. Even in the females, responses were only just greater than in the controls. More marked effects were observed in the animals treated with wax. These showed responses intermediate between those evoked by Tiger Balm White and by Tiger Balm Red.

Hyperkeratotic responses, with and without inflammatory changes, were most common in rabbits treated with Tiger Balm Red. Changes were not totally consistent between males and females or within a group. For example, it was possible, with Tiger Balm Red treatment, to find the occasional skin that was entirely normal. Moreover, although Tiger Balm Red produced the most marked responses, the changes were relatively mild and there was no suggestion of severe skin damage or necrosis.

Discussion

Tiger Balm marketed in Europe and the USA carries the following directions: "rub gently on affected parts, for external use only; keep out of reach of children and avoid contact with mucous membranes". Skin irritancy possibly caused by Tiger Balm can be considered in relation to these directions.

Applications of Tiger Balm Red or Tiger Balm White to the clipped skin of New Zealand strain white rabbits for 21 days did not produce any marked systemic toxicity, although skin irritation occurred. The skin irritation produced by Tiger Balm Red was more marked than that produced by Tiger Balm White; the latter was almost innocuous in male rabbits. The irritation produced by Tiger Balm was not progressive, however, and tolerance developed within 10 days. Furthermore, even with maximum irritation, no severe damage to the treated skin was detected by skin histopathology even after the 21-day treatment. The sensitivity of the rabbit's skin was shown by the fact that a mixture of a commercial hard wax and a soft wax applied to the skin in the same manner as the Tiger Balm produced recordable reactions.

With Tiger Balm Red the level of irritation was within that which is arbitrarily described as being serious. This was perhaps not unexpected, since Tiger Balm is designed to be a rubefacient and counter-irritant and therefore contains irritants such as clove oil, menthol and camphor.

When irritants produce only hyperaemia they can be classified as *rubefacients*, whereas *vesicants* cause capillary damage to the point of permeability changes and exudate formation. A further level of dermal damage is the formation of multiple small abscesses, as occurs with *pustulants* (Swinyard & Pathak, 1980). By such criteria Tiger Balm Red would appear to have a rubefacient action in rabbits. Camphor is well recognized for its rubefacient and mild local-anaesthetic actions and can be used as a 10% solution in alcohol (*National Formulary*, 1970).

Other components of Tiger Balm have also been recognized as having some rubefacient action. In particular, clove oil is a powerful rubefacient and a protoplasmic poison (Tyler, Brady & Robbers, 1975). A comparison of the components of Tiger Balm Red with those of the White shows that both contain cam-

Table 2. *Histopathology scores for skin samples taken from rabbits treated topically for 21 days with Tiger Balms Red and White*

		Mean scores*			
		Treated areas		Untreated (control) areas	
Treatment	Sex	Abraded	Unabraded	Abraded	Unabraded
Tiger Balm Red	M	2·3 ± 0·3	2·4 ± 0·3	1·3 ± 0·3	1·0
	F	2·3 ± 0·3	2·1 ± 0·3	1·4 ± 0·3	1·1 ± 0·1
Tiger Balm White	M	1·7 ± 0·4	1·6 ± 0·2	1·1 ± 0·1	1·3 ± 0·3
	F	2·0 ± 0·3	1·9 ± 0·3	1·4 ± 0·3	1·4 ± 0·3
Wax	M	1·8 ± 0·1	2·3 ± 0·3	1·1 ± 0·1	1·0
	F	2·0 ± 0·3	1·9 ± 0·3	1·0	1·0

*Scores (expressed as means ± SEM for sections from groups of seven rabbits) were derived from 'blind' examination of the sections using the following scoring system: 1 for normal skin, 2 for hyperkeratosis and 3 for hyperkeratosis with inflammatory changes. No higher scores were needed. No statistically significant differences between the groups could be identified in treated skin, whether abraded or unabraded. Scores for untreated skin (abraded or unabraded) were lower than those for treated skin to a statistically significant degree ($P < 0.05$ by analysis of variance—Anova).

phor, menthol and cajuput oil in similar proportions, but that the major difference is in the concentration of clove oil, which in Red is 2·5 times that in White (Table 1). The proportions of the different waxes in Tiger Balm Red were also slightly different from those in White.

In general, rabbit-skin irritancy tests are very sensitive and are good predictors of human responses. The sensitivity of the test is such that a variety of relatively innocuous substances will produce reactions on rabbit skin. For example, in 21-day continuous patch tests involving a variety of substances at their threshold concentrations, average scores of up to 3·0 were obtained; scores greater than 2·0 were obtained with agents as diverse as diethyltoluamide and salicylic acid (Marzulli & Maibach, 1975; Steinberg *et al.* 1975).

The more vigorous response encountered with Tiger Balm Red requires explanation. It was possibly due to the different components of the Red formulation, namely the greater clove oil content and the presence of cassia oil and ammonia solution. However, the ammonia in Tiger Balm Red reacts with cassia oil to produce the formulation's characteristic colour and, furthermore, published information indicates that cassia oil does not have markedly irritant properties (*British Pharmaceutical Codex*, 1973; *Martindale*, 1977). On the other hand, clove oil is a well-known irritant capable of producing tissue damage.

The histopathological findings correlate well with the Draize scores. The most marked changes were encountered with Tiger Balm Red, although these changes did not include severe skin damage and/or necrosis. Changes were minimal when compared with the necrotic changes that would be encountered on application of a vesicant or pustulant. Overall, histological changes were consistent with a mild defence reaction to continuous application of a rubefacient. If one considers the histological changes to have been essentially protective, then the tolerance that developed with prolonged treatment could have been due to the hyperkeratotic response. These changes were only pertinent to Tiger Balm Red, for changes with Tiger Balm White were hardly significant; this accords with the defence-reaction postulate, for Tiger Balm White had very limited rubefacient action.

Skin application of Tiger Balm did not produce any systemic toxicity or any signs of the general toxicology that would follow parenteral or oral administration of the major components of Tiger Balm (Table 1).

Conclusion

The common proprietary rubefacient Tiger Balm White is relatively innocuous when applied to the skin of rabbits. Tiger Balm Red, either because of an extra ingredient or higher concentrations of common ingredients, has a more marked rubefacient action to which tolerance develops. The relevance of these findings to any clinical observations on Tiger Balm are not known.

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Review Section

PLASTICIZER MIGRATION FROM POLYVINYL CHLORIDE FILM TO SOLVENTS AND FOODS

D. E. TILL
Arthur D. Little, Inc.

R. C. REID
Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

P. S. SCHWARTZ
Food and Drug Administration, Washington, DC 20204

and

K. R. SIDMAN, J. R. VALENTINE and R. H. WHELAN
Arthur D. Little, Inc., Acorn Park, Cambridge, MA 02140, USA

(*Received 11 June 1981*)

Summary—Polyvinyl chloride (PVC) films used for food wraps contain significant concentrations of plasticizers, along with other additives. The rate of migration of these plasticizers to foods and food-simulating solvents is the principal concern of this paper, which reviews prior experimental studies and presents new data for radiolabelled dioctyl adipate. Analytical models are described to correlate many of the data, criteria are presented for identifying the controlling step in the mechanism of transfer of plasticizer from PVC films into foods and food-simulating solvents, and tentative recommendations are offered for the selection of food simulants and for the type of experiment necessary to allow an unambiguous interpretation of the data.

Introduction

Plasticized PVC film is widely used as a food wrap because it is flexible and transparent and has high permeability to oxygen. Typically such films are less than 25 μm (1 mil) thick and are used for packaging fresh meat and produce. The formulation of a typical commercial film wrap which is used for meat and in which dioctyl adipate is the primary plasticizer, is presented in Table 1.

In food packages, a plasticizer has the potential to migrate from the wrap into food and thereby become an indirect food additive. Because of this, the US FDA regulates which plasticizers may be used in food wraps and sets the limits for their allowable migration. It is the responsibility of industry (usually the plasticizer manufacturer) to demonstrate that each food wrap formulation is in compliance with the FDA regulations for the foods that are to be packaged with the wrap. Experimentation with actual foods is difficult because of the analytical problems in quantifying small concentrations of migrating species. Furthermore, the varieties and numbers of foods make comprehensive testing virtually impossible. In recognition of these factors, the FDA specifies that testing may be conducted with solvents that simulate food groups. The specific solvents are: distilled water, 3% acetic acid, 8 and 50% ethanol and *n*-heptane. In addition, accelerated migration studies may be carried out with these solvents at elevated temperature.

There is a question as to whether the simulation test results predict the behaviour of the wraps in contact with foods. To provide reliable, yet conservative, correlations between the migration of additives into foods and into simulating solvents, the FDA and governmental regulatory agencies in several other nations are actively involved in experimental programmes that address a variety of polymers and additives.

This paper is concerned only with the loss of the principal plasticizer from PVC into various solvents and foods. It reviews the previous literature on this problem and presents new data. Of primary interest is the mechanism of plasticizer loss in various systems so that generalizations can eventually be developed that relate to many solvent/food/polymer combinations.

Prior experimental studies

Several studies involving particular PVC formulations identified phenomena that apply in general to the process of plasticizer migration. Besides the use of plasticized PVC as a food wrap, similar formulations

Abbreviations: DBP = Dibutyl phthalate; DOA = dioctyl adipate; DOP = dioctyl phthalate; FDA = Food and Drug Administration; phr = parts per hundred of resin.

Table 1. *Typical commercial meat-film PVC wrap*

Ingredient	Function	Concn (% w/w of total formulation)
PVC homopolymer	Base resin	65–70
Dioctyl adipate	Primary plasticizer	20–25
Epoxidized soya-bean oil	Secondary plasticizer	3–10
Glycerol and sorbitan mono-oleates and polyoxyethylene derivatives	Antifogging agents*	1–5
Calcium/zinc stearate	Thermal stabilizer	0·3–1·5
Stearic acid	Release agent	0·2–1·0
FDC toner	Colour improver	<1

*To prevent formation of beads of moisture on film.

are often used to coat fabrics for upholstery, clothing and other applications where flexibility is important. The loss of plasticizer into air then leads to a degradation of product quality. To study this problem, Small (1947) carried out a number of experiments using PVC plasticized with dibutyl phthalate (DBP). Film strips were hung in a moving warm air stream. Over short periods, the quantity of DBP evaporated was linear with time, but over long periods, this proportionality was no longer valid. As will be discussed later, these results suggest that the initial rate was limited by mass transfer in the air, but at longer times, the diffusional resistance within the film became important.

In a pair of papers, Quackenbos (1954) and Reed, Klemm & Schultz (1954) reported upon the migration of a number of different plasticizers from PVC. Some experiments involved measurements of plasticizer losses in air. As in the Small (1947) study, the initial plasticizer loss was noted to be linear with time. In fact, the migration data for the polymer films were not greatly different from those wherein a glass-cloth was wetted with the same plasticizer. Again the conclusion reached was that initial rates were controlled by mass transfer resistances external to the film. However, when the same experiments with polymer films and wetted glass-cloth were repeated in an evacuated chamber, loss rates increased but those from the cloth exceeded those from the film.

Plasticizer migration into liquids was studied using mineral oil and 5% soap solutions. In these cases, the mass that migrated was found to correlate well with the square root of time and the conclusion was drawn that, for these liquids, diffusion within the polymer was the rate determining step. Diffusion coefficients for dioctyl adipate (DOA) and dioctyl phthalate (DOP) were estimated and are shown in Fig. 1 as a function of temperature. These data were obtained from mineral-oil studies, but similar results were reported for 5% soap solutions and high-vacuum tests. In all cases the initial plasticizer content was quite high, i.e. greater than 50 phr.

A profound effect was found relative to the initial concentration of plasticizer. For concentrations in the range of 0–20 phr, rates of migration were small and almost independent of plasticizer content. At higher plasticizer concentrations, the rate of migration rose more rapidly than would have been expected if a linear correlation with concentration were assumed.

In a study at Badische Anilin- und Soda-Fabrik AG, Pfab (1973) measured the plasticizer-concentration profiles in PVC films after extraction, using DOP as the plasticizer in an initial concentration of about 52 phr. The solvents used were sunflower and coconut oils. DOP migrated *out* of the film, the oils migrated *in*. The data could be correlated assuming that both diffusion processes were independent. With a simple one-dimensional Fickian analysis, described later in this paper, diffusion coefficients for both DOP and the oils were estimated to be about 5×10^{-10}–10^{-9} cm^2/sec at 40°C. At lower temperatures the diffusion coefficients decreased, but they did not follow an Arrhenius relationship.

In a set of five papers, Kampouris and his colleagues (Kampouris, 1975a, b & 1976; Kampouris & Papaconstantinou, 1976; Kampouris, Regas, Rokotas *et al.* 1976) presented results on the migration of DBP and DOP from PVC films. The solvents included edible and mineral oils, alcohols and aqueous ethanol. In most cases, the PVC (prepared by suspension polymerization) had an average molecular weight

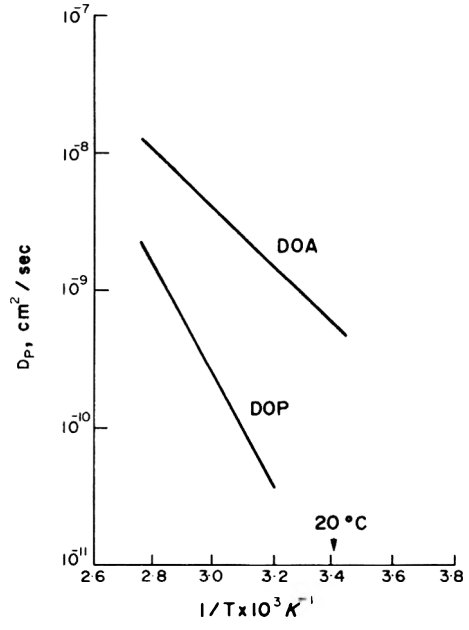

Fig. 1. Approximate values of diffusion coefficients of plasticizers in PVC containing an initial concentration of dioctyl adipate (DOA) or dioctyl phthalate (DOP) of approximately 60 phr derived from studies of migration into mineral oil (source: Quackenbos, 1954).

of about 62,000. In one case, PVC films with a slightly higher molecular weight (mean 90,000) were also studied (Kampouris, 1975b; Kampouris *et al.* 1976). Significantly higher migration rates were recorded for the lower-molecular-weight film.

The effect of initial plasticizer concentration was found to be very significant, i.e. the higher the concentration, the faster the migration. As illustrated in Fig. 2, showing DBP extraction from PVC film into olive oil at 30°C, little DBP migrated (2% of the total in 80 days) when the initial concentration was low (e.g. c. 20 phr). With an initial concentration of 40 phr, 12% was lost in 80 days and, with 60 phr, 25%.

In the Kampouris work other variables were also studied, and an increase in temperature was found to result in more rapid migration. Also, Kampouris & Papaconstantinou (1976) explored the migration of DOP and DBP from PVC in cases where a portion of the plasticizer was replaced by an edible oil. When this was done, there was a significant enhancement of the loss of the DOP or DBP, and this was especially true if the solvent oil and the substitute oil were one and the same.

In one of the few experimental studies of plasticizer migration from PVC to foods, Daun & Gilbert (1977) used a DOA-plasticized PVC wrap on meat and fat. The film had an original DOA content of 42 phr. The meat/meat fat was ground and shaped into a pattie about 2·5 mm thick and both sides were covered by a film backed by weighted glass plates. The results indicated that about 15 mg DOA/dm^2 had migrated into low-fat meat after 72 hr at 4°C but, for the high-fat meat, the value rose to about 22 mg/dm^2. This latter value represented a significant fraction (*c.* 50%) of the original DOA in the film.

Theory of migration process

The migration of plasticizer from PVC films to solvents or foods can be visualized as a two-step process. Within the polymer, migration occurs by a diffusive process which is generally considered to be Fickian in nature. At the interface between solvent and polymer, there is assumed to be a discontinuity in concentration as the migrant is partitioned between the two phases. In the solvent or food phase, the mechanism of migrant transport depends upon the physical properties of this phase. If the phase is immobile, as in a solid food, then diffusive transport occurs. If the external phase is a fluid, then movement of the migrant away from the polymer is normally controlled by convective mixing processes. In both cases, migrant transport rates are reduced as the external phase approaches equilibrium. A general discussion concerning the effect of the external phase is treated elsewhere (Reid, Sidman, Schwope & Till, 1980). For the purposes of the present work, we are interested in three specific cases which may be used to describe most of the experimental test data on plasticizer migration from PVC films.

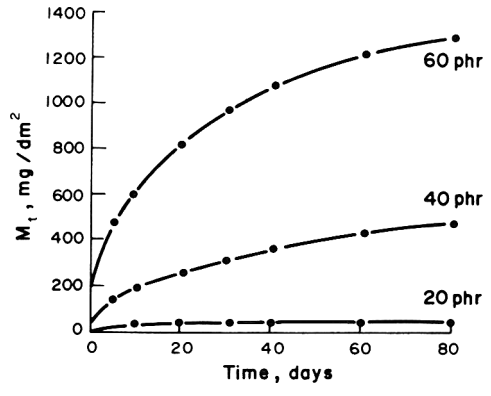

Fig. 2. Migration of dibutyl phthalate into olive oil at 30°C from PVC containing an initial plasticizer concentration of 20, 40 or 60 phr (source: Kampouris, 1976).

The simplest model assumes that the solvent is well mixed and never saturated with plasticizer. Thus the rate determining step is that for the diffusion of plasticizer from within the PVC film to the surface. If we assume further that the plasticizer is, initially, evenly distributed in the film at a concentration of C_{p_0}, and that the diffusion coefficient of the plasticizer in the polymer may be treated as a constant, D_p, then, as shown by Crank (1975):

$$M_t = 2C_{p_0}(D_p t/\pi)^{\frac{1}{2}} \quad (1)$$

M_t being the total plasticizer lost from the film in time t. Equation (1) also assumes that the film is sufficiently thick for the concentration of plasticizer at the mid-plane (for two-sided extraction) to remain at its original value, C_{p_0}. Physically this would be achieved if less than about 15–20% of the plasticizer were lost. [More complex expressions are available for cases where more plasticizer is removed (Crank, 1975).] Equation (1) indicates that migration is proportional to the square root of the elapsed time and this relationship has been noted earlier. One of the principal criticisms directed toward this simple model is that the diffusion coefficient of the plasticizer most probably varies with plasticizer concentration, and thus is likely to be applicable in cases where only small amounts of plasticizer are removed or when solvents penetrate the film and simulate the plasticizer that has been lost. For example, in the studies of Pfab (1973), DOP was the plasticizer and both coconut and sunflower oils were used as solvents. As DOP migrated out of the film, the oils migrated in. Pfab (1973) measured the concentration profiles of both the solvent oil and DOP as a function of thickness and time. He found that equation (1) would correlate both the migration of DOP away from the film and the diffusion of oils into the film. Quackenbos (1954) also used equation (1) to correlate his data for plasticizer migration into mineral oils and soap solutions.

Effect of agitation

The model leading to equation (1) may be modified to include a mass transfer resistance on the solvent side. In this case, the bulk solvent is assumed to be well mixed (and infinite in its capacity to accept migrant without becoming saturated), but at the interface on the solvent side, the rate of transfer of migrant is given by

$$\frac{\text{Rate}}{\text{Area}} = k(C_{s_i} - C_{s_\infty}). \quad (2)$$

where C_{s_i} is the concentration of the migrant at the

PVC film interface while C_{s_1} is the migrant concentration in the bulk solvent. The mass transfer coefficient k may be estimated by standard engineering correlations and depends on the fluid turbulence at the boundary. [k would approach infinity for the well-mixed case described by equation (1).]

This case is discussed by Crank (1975) and his results may be rearranged in the form given by equation (3):

$$M_t = 2C_{p_0}(D_p t/\pi)^{\frac{1}{2}}[1 + (\sqrt{\pi}/2Y)(e^{Y^2}\,\mathrm{erfc}\,Y - 1)] \quad (3)$$

M_t is again the total quantity of plasticizer lost in time t. The parameter Y is given as

$$Y = kK(t/D_p)^{\frac{1}{2}} \quad (4)$$

where K is the partition coefficient and represents the ratio of plasticizer concentration in the solvent to that in the PVC film at equilibrium. Under conditions where Y is large (large k, large K, long time, low D_p), equation (3) reduces to equation (1), i.e. the migration is proportional to the square root of time. At very small values of Y (e.g. over short periods), equation (3) simplifies to

$$M_t \simeq kKC_{p_0}t \quad (5)$$

where it has been assumed that C_{s_1} is small. Thus, in this period the migration is proportional to time. These two time dependencies have been noted by Small (1947) and Quackenbos (1954), as described earlier, for plasticizer loss from PVC into air and flowing water. The linear time period corresponds to an external mass transfer process as the rate determining step while the square root of time period indicates that diffusion of the plasticizer from the interior of the film is the rate-determining factor.

The model given by equation (3) may also be used to describe the experiments on plasticizer loss carried out by Kampouris (1975a), who stated that his migration data would correlate with neither t nor $t^{\frac{1}{2}}$. In Fig. 3 we show some of his results plotted as mass of the plasticizer (DBP) migrated as a function of the square root of time. Three time periods can be distinguished. Over a short time (<2 days), the loss of DBP varies with t^n where $n > \frac{1}{2}$. Then for a week or more, the migration is proportional to $t^{\frac{1}{2}}$ and, at longer times the rate drops off. These trends can be rationalized in a qualitative manner by assuming that, initially, external mass transfer is rate controlling. At intermediate times, diffusion of the DBP within the polymer controls and, finally, at long times the depletion of DBP within the polymer begins to affect the rate. Expanding upon the last regime, one notes that the results illustrated in Fig. 3 were for films that were 2 mm thick and contained, initially, about 900 mg DBP/test sample. Since the area of transfer was 0·20 dm^2, then about 15% of the DBP had migrated when $M_t \sim 670\ mg/dm^2$. Quackenbos and others have shown that the migration rate begins to tail off when 10–20% of the migrant has been lost. The tailing off of Kampouris' data agrees with this criterion.

To apply equation (3) to these data, we have selected values of $D_p = 7 \times 10^{-11}\ cm^2/sec$ and $kK = 9 \times 10^{-9}\ cm^2/sec$ and have compared the M_t value from equation (3) with experimental data for lubricating oil A (Fig. 4). There is good agreement for the first 50 days. The values of both D_p and kK appear to be too small for this particular system and should not be judged reliable. The important point, however, is to note that the simple diffusion model, when modified for an external mass transfer resistance can predict the observed linear time period of migration at short times—and it is an experimental fact that such a period exists.

The final model of interest here is similar to that which led to equation (1), but instead of assuming that the solvent (food) phase is well mixed, we select it to be immobile so that migration into this phase must occur by diffusion. Thus, we must introduce a solvent phase diffusion coefficient into the migration equation.

The result is:

$$M_t = 2C_{p_0}(D_p t/\pi)^{\frac{1}{2}}[\beta/(1 + \beta)] \quad (6)$$

We note that equation (6) is identical to equation (1) except for terms containing the parameter β.

$$\beta = K(D_s/D_p)^{\frac{1}{2}} \quad (7)$$

K is the equilibrium partition coefficient described earlier and D_s represents the diffusion coefficient of the plasticizer in the immobile solvent (food) phase. If β is large, then equation (6) reduces to equation (1), i.e. the rate-determining step is the diffusion of the plasticizer within the PVC. If β is very small then

$$M_t = 2C_{p_0}K(D_s t/\pi)^{\frac{1}{2}} \quad (8)$$

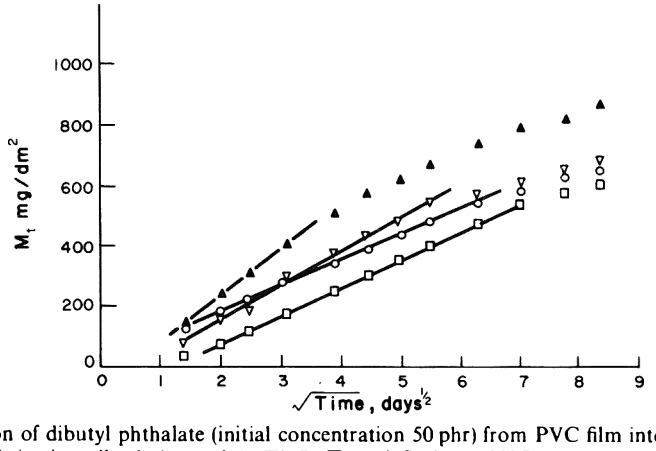

Fig. 3. Migration of dibutyl phthalate (initial concentration 50 phr) from PVC film into paraffin oil (○) or one of the lubricating oils, designated A (□), B (▽) and C (▲), at 30°C (source: Kampouris, 1975a).

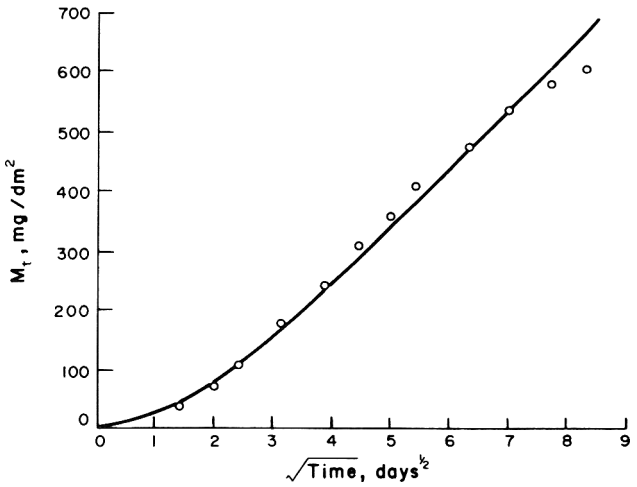

Fig. 4. Comparison between experimental data (from Kampouris, 1975a) for the rate of migration of dibutyl phthalate from PVC into lubricating oil A at 30 C and the migration rate calculated from $M_t = 2C_{p_0}(D_p t/\pi)^{\frac{1}{2}}\ [1 + (\sqrt{\pi}/2Y)(e^{Y^2}\text{erfc}Y - 1)]$, using values of $D_p = 7 \times 10^{-11}$ cm^2/sec, $Kk = 9 \times 10^{-9}$ cm/sec and $C_{p_0} = 0{\cdot}4$ g/cm^3.

and the rate-determining step is diffusion within the solvent (food). We shall use equation (6) later when discussing generalized correlations.

Effect of temperature and initial plasticizer level

We have suggested that equation (3) may be used to estimate the migration of plasticizer from PVC into liquid solvents.

If the solvent is stirred vigorously or if the partition coefficient is large, equation (3) reduces to equation (1). In the latter case there is one undetermined parameter, the diffusivity of the plasticizer in the PVC, D_p, which will depend upon the properties of the plasticizer and upon the history of the PVC. This generalization has, nevertheless, one important limitation. At low initial concentrations of plasticizer, migration rates may be vanishingly small. Even at 40°C, Pfab (1973) found no measurable migration of DOP into coconut or sunflower oils when the initial concentrations were either 5 or 15% by weight (5–18 phr). Quackenbos (1954) and Reed *et al.* (1954) rarely worked with less than 40 phr. The Kampouris papers (1975a,b; 1976) show data for 20 phr in some cases, but the rates of migration of both DBP and DOP were very low.

Pfab (1973) suggests that diffusion can occur much more rapidly within a polymer if the temperature is significantly above the glass-transition temperature, T_g. He then shows that T_g decreases with an increase in plasticizer concentration. If $T_g \sim T$, then the diffusivity of the plasticizer may drop by orders of magnitude with small changes in temperature. In addition, he uses the same argument to explain the enhancing effect of an increase in temperature on the rate of migration—at temperatures above T_g the polymer lattice is more flexible and diffusion occurs more rapidly. In Fig. 5 we show his correlation for the diffusion coefficient of DOP in PVC films as a function of $T - T_g$. The test temperatures and DOP concentrations vary widely but the correlation with $T - T_g$ is excellent.

New data

The prior work and physical/mathematical models discussed above provide the background necessary for correlating plasticizer migration into foods and food simulants. In the sections that follow, we describe our study, which involved measuring the rate of migration of radiolabelled DOA plasticizer from PVC films into foods and food-simulating solvents.

Experimental

Test materials. The radiolabelled DOA was synthesized from [^{14}C]adipoyl chloride and 2-ethylhexanol. Final purification was achieved by vacuum distillation. Radiochemical purity was found to be 98·6% as measured by thin-layer chromatography. The specific activity was 4·84 mCi/g. The PVC compound was pre-

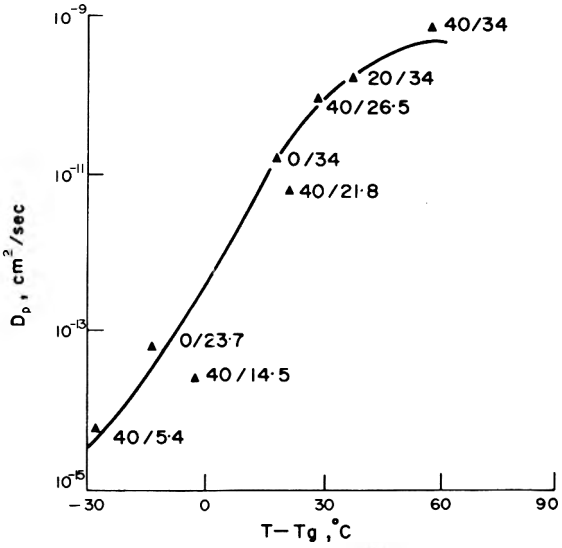

Fig. 5. Correlation (from Pfab, 1973) of the diffusion coefficient of dioctyl phthalate (DOP) in PVC films with the excess of temperature over glass-transition temperature (T − Tg). The two values indicated at each data point are the temperature (T; °C)/DOP content (%) of PVC.

pared by adding [^{14}C]DOA to a base formulation supplied by a commercial manufacturer. The base formulation was similar to that shown in Table 1 except that only about one-half of the normal DOA concentration was used. The average molecular weight of the PVC was 148,000. A mixture of both radiolabelled and unlabelled DOA was added to this base compound to raise the total plasticizer content to about 24%, w/w (32 phr). The mixture was fusion-blended in a Brabender Plasticorder at 160°C and 60 rev./min for 5 min. Films were hot-pressed from small portions of this masterbatch; the final thickness being about 66 μm and the density 1·2 g/cm^3. Portions of each pressing were analysed for uniformity of dispersion of the [^{14}C]DOA, to verify the specific activity of the film, and to determine the total DOA content. The final film had an activity of about 1·1 mCi/g DOA or 0·26 mCi/g film.

Migration into foods. The foods tested were ground beef, beef fat, lean beef, chicken breasts, fish fillets, apples and carrots. The fish, chicken and beef products were each placed upon foil-coated wooden blocks. The top surface of the food was covered with a 1-dm^2 disc of the radiolabelled film and was overwrapped with a commercial PVC film. The unit was then inverted and stored at 4°C. Apples and carrots were wrapped with 1 dm^2 of the radiolabelled PVC film, overwrapped with non-radioactive film and also stored at 4°C. Prior to analysis, the apples and carrots were trimmed to remove all portions that had not been in contact with the test film.

To determine the [^{14}C]DOA in the food, the foods were homogenized with water and the samples were combusted. The CO_2 was then trapped, diluted with ACS scintillation fluor (Amersham/Searle Corp., Arlington Heights, IL) and counted.

Migration into solvents. The food-simulating solvents used in the programme were those specified by the US FDA, namely water, 3% acetic acid, 8 and 50% ethanol and *n*-heptane. In addition, we investigated the use of corn oil as a simulant for fatty foods in general.

Two techniques were used to measure the migration of DOA into the test solvents. In the first or 'sandwich' method, either one or three circular specimens of film were held horizontally in stainless-steel screens and placed in a vial of solvent. In the three-layer test, the ratio of solvent volume/film area (V/A) was 0·31 cm^3/cm^2 while for the single-layer experiments the ratio was 1·55 cm^3/cm^2. No agitation was used, but, before sampling, the vials were swirled. In the second method, films were stretched on frames and placed in jars, and solvent was added. The jars were mounted on ball-mill rolls and rotated in an end-over-end manner at either 6 or 12 rev./min. The V/A ratio was 1·8 cm^3/cm^2. The temperature for most tests was 21°C.

Results of food tests

A summary of the migration results for the foods is given in Table 2. A correlation of migration with the fat content of the foods was observed. Beef fat showed the highest levels of DOA and chicken breasts the next highest. (The chicken breasts had a fatty skin and autoradiography later showed that the DOA migration was confined to this skin.) Less DOA was found in lean beef and fish while very little migrated into the apples or carrots.

The results for ground beef are similar to the Dawn & Gilbert (1977) results described earlier. In other tests, chicken breasts were tested at 2 and 4 days in addition to the 7-day results shown in Table 2. As shown later, the 2-, 4- and 7-day migration data correlated reasonably well with the square root of time.

Results of solvent tests

Figures 6–8 summarize the effects of three independent variables—solvent type, solvent loading (cm^3 solvent/cm^2 film) and agitation—on DOA migration.

Effect of solvent type. Figure 6 shows the migration obtained using the sandwich cell at a temperature of 21°C and a solvent loading of 1·55 cm^3/cm^2 film. This temperature was selected because it is the exaggerated condition specified by the FDA for the evaluation of films to be contacted with refrigerated foods. The figure shows that heptane had rapidly extracted essentially all of the DOA in the film (*c.* 100 mg/dm^2) by day 3, when the first measurement was made. Corn oil extracted DOA at a slower rate than heptane, and after 12 days approximately 70% of the initial plasticizer had been extracted. With 50% ethanol an equilibrium DOA concentration was reached within 3 days, but this level was less than half that observed with corn oil. In contrast, the aqueous solvents (water, 3% acetic acid and 8% ethanol) extracted only a small

Table 2. *Results of tests of migration of dioctyl adipate (DOA) from PVC film (1 dm^2) into food over a 7-day period at 4°C*

Food	Fat content (%)	Migration of DOA* mg/dm^2	Migration of DOA* mg/g film
Beef fat	79	29	36
Chicken breasts	36†	19	20
Ground beef	22	12	14
Fish fillet	0·14	2·4	2·9
Lean beef	4·4	1·4	1·8
Apples	0·53	0·22	0·3
Carrots	0·25	0·044	0·06

*The original film contained about 32 phr or 240 mg DOA/g film. With one-sided exposure and a film thickness of 66 μm, the migration of 240 mg DOA/g film would correspond to about 180 mg/dm^2.

†Analysis of skin only.

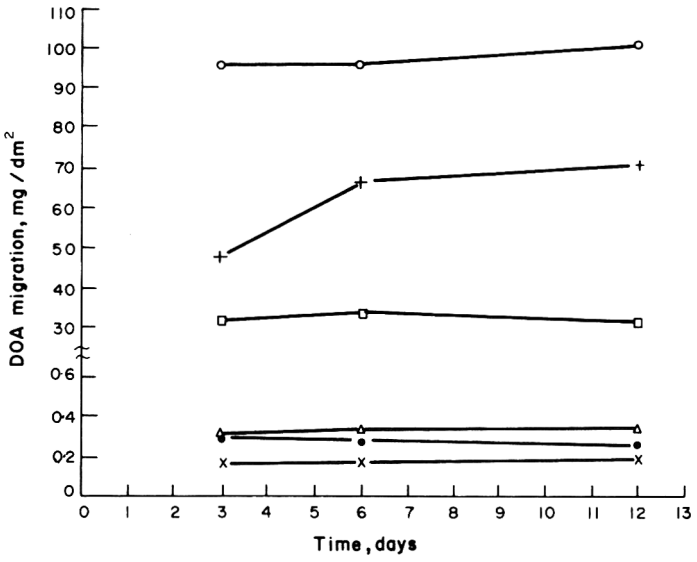

Fig. 6. Effect of solvent type on dioctyl adipate migration from PVC film in a sandwich cell at 21°C, using a solvent loading of 1·55 cm^3/cm^2 film for heptane (○), corn oil (+), 50% ethanol (□), 8% ethanol (△), water (●) and 3% acetic acid (×).

fraction of the plasticizer content of the film. (Note the scale change in the figure.)

Effect of solvent loading. We evaluated loadings of 0·31 and 1·55 cm^3/cm^2 as shown in Fig. 7. Except in the case of 8% ethanol, the higher solvent loading led to enhanced migration of DOA. This behaviour is consistent with the low solubility of DOA in the aqueous solvent, and it points to a problem with respect to the FDA-prescribed extraction methodology. In situations where the simulating solvent offers measurable resistance to the transport of DOA (e.g. because of low DOA solubility or high viscosity of the solvent), one must consider the possibility that the migration test apparatus itself can influence the results. Such factors as film spacing, screen construction, film orientation (i.e. horizontal, vertical, angled), degree of agitation and uniformity of temperature distribution can all be important. Current extraction methods such as the sandwich procedure allow considerable latitude in the conduct of the test, and may lead to significant inter-laboratory differences in migration data.

Effect of agitation. Figure 8 provides a comparison of DOA migration under unagitated and agitated

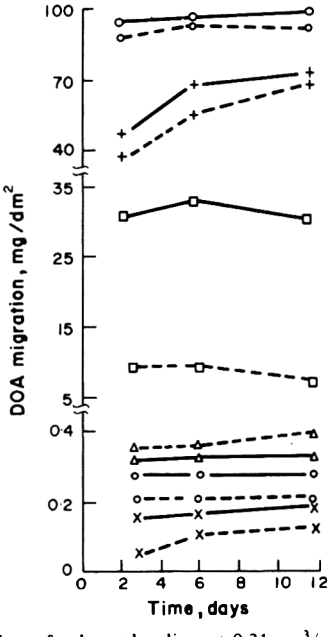

Fig. 7. Effect of solvent loading at 0·31 cm^3/cm^2 (– – –) and 1·55 cm^3/cm^2 (——) on dioctyl adipate migration from PVC film into heptane (○), corn oil (+), 50% ethanol (□), 8% ethanol (△), water (●) or 3% acetic acid (×) in a sandwich cell at 21°C.

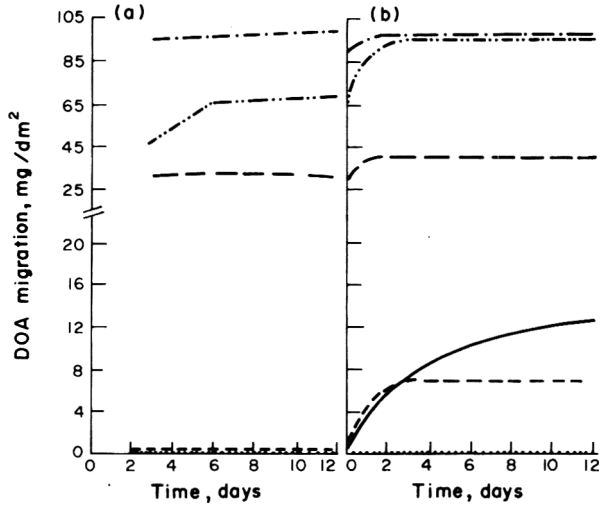

Fig. 8. Migration of dioctyl adipate from PVC film into heptane (—·—), corn oil (—··—), 50% ethanol (——), 8% ethanol (– – –), water (——) or 3% acetic acid (·····) in a system that was either (a) not agitated or (b) agitated by end-over-end rotation at 6 rev./min. The traces for 8% ethanol, water and 3% acetic acid in the unagitated system, like that for 3% acetic acid in the agitated system, lie close to the base line.

conditions. Migration into heptane was essentially unaffected by agitation; the DOA was rapidly and completely extracted from the film. In the case of corn oil, agitation increased the amount of DOA that migrated by approximately 50% over the unagitated level—thereby completely extracting all the plasticizer. Agitation had no effect on the 50% ethanol results, presumably because equilibrium was rapidly established, even in the unagitated case.

The effect of agitation on the results with water and with 8% ethanol was dramatic. At 6 rev./min, the amount of DOA migrating reached levels that were more than ten times higher than those achieved in the unagitated cases. Analyses of DOA concentrations in these solvents (Table 3) revealed that the saturated solubility levels had been exceeded. During the tests the solutions became cloudy, suggesting that a suspension had formed. The ability of plasticizers to migrate into aqueous solutions that have previously been saturated with them is well documented in the literature (see Quackenbos, 1954). However, the mechanisms are poorly understood.

In contrast to the water and 8%-ethanol results, the DOA migration into agitated 3% acetic acid was low. We can only speculate on the reasons for the conflicting behaviour of these aqueous solvents. It is believed to involve either the co-migration of plasticizer with other ingredients in the film, the counter-migration of water, or a difference in the interfacial surface tension between the DOA and solvents. However, the principal objective of this study was to relate DOA migration into simulants with its migration into foods. This is discussed below.

Simulation of plasticizer migration into food

In selecting a simulant to mimic food, one should first attempt to describe the food. Is it a solid, liquid or emulsion under normal storage conditions? Does it possess components that might be soluble in the PVC? If so, what are their concentrations and are they bound in any chemical way to the food? These, and similar questions, however, are seldom raised. Foods are normally complex materials with ill-defined structures and compositions. The usual classification of foods involves such vague terms as 'fatty in nature', 'aqueous' and 'oil-in-water' (or *vice versa*) emulsion. Yet even these descriptive phrases are often helpful.

Aqueous foods

Consider an 'aqueous' food. This expression implies that the fat or oil content is very low. The carrots and apples tested in the present work would be examples of such foods. In these cases, water would be expected to be a suitable simulant. The fact that water is a fluid while many (if not most) aqueous-type foods are solid does cause concern, however, as PVC-plasticizer solubilities in an aqueous medium are very low, and, as noted earlier, concentration levels may even exceed saturation values. Also, the external mass transfer resistance may be important and migration rates into water become sensitive to extraction cell design and degree of agitation. Solid aqueous foods would not present such problems as plasticizer transfer from the film into the food must occur by a diffusion process and equation (6) could be used. As noted earlier, if the parameter β is large (due to a large value of K or to a small value of D_p), then the diffusional resistance in the food is negligible and equation (6) reduces to equation (1). On the other hand, for aqueous foods K is probably quite low; K for 3% acetic acid was estimated to be 7×10^{-6} and for water *c.* 10^{-5}. For small β, equation (6) reduces to equation (8), i.e. the principal resistance to migration now resides in the food (or solvent) phase. As an example, if $K \sim 10^{-5}$, $D_s \sim 10^{-6}\,cm^2/sec$ and $D_p \sim 10^{-12}\,cm^2/sec$, $\beta = 0{\cdot}01$. In this case, equation (8) would be applicable and a suitable simulant for the aqueous food would be water. However, the extraction experiments would then have to be carefully planned to minimize turbulence in the aqueous phase so as to limit the migration process to one of diffusion. In fact, if these values of K and D_s are assumed appropriate for apples and are used in equation (8), with $C_{p_0} \sim 0{\cdot}30\,g\ DOA/cm^3$, then, after 7 days, the amount of DOA migrated to the apples is $0{\cdot}26\,mg/dm^2$. This is in excellent agreement with the experimental value given in Table 2. Carrots should also represent the case of an aqueous food and the migration after 7 days would have been expected to be similar to apples whereas it is, in fact, much lower. We suspect that the irregular surface of the carrot may have prevented good contact between the PVC film and the carrot.

The tentative conclusion reached from the few studies made to date with plasticizer migration from PVC to aqueous solid foods is that water may be used as a simulant, but an experimental extraction cell that prevents convective mixing should be used. The total

Table 3. *Concentrations of dioctyl adipate (DOA) in solution following agitation of plasticized PVC film in water or 8% ethanol at 6 rev./min for 2–12 days*

Solvent	Saturation solubility at 21 C* ($\mu g/cm^3$)	Solution concentration ($\mu g/cm^3$) by day		
		2	5	12
Water	15	13	55	73
8% Ethanol	12	39	36	41

*Determined by dispersing 15 mg radiolabelled DOA in 15 cm^3 of the solvent, allowing the droplets to coalesce in a separatory funnel for 5 days, and then drawing off solvent for analysis.

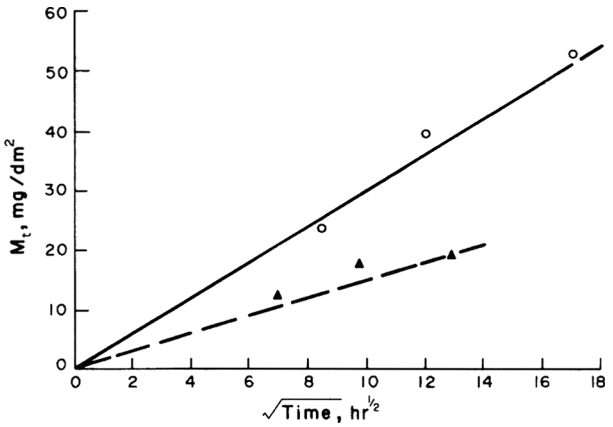

Fig. 9. Migration of dioctyl adipate from PVC film into quiescent corn oil (○) and into chicken-breast skin (▲) in a sandwich cell at 4°C. Predicted migration into chicken-breast skin is indicated by a broken line.

migration should be linear with the square root of time, as shown in equation (8). The data would then allow an estimation of the product $K\sqrt{D_s}$ and, if several temperatures are used, this product may be correlated with temperature to predict the degree of migration at other temperatures. Changes in the migration due to small variations in the initial plasticizer loading may also be studied with equation (8), but for large variations, experiments may be desirable to prove that β [in equation (6)] is still small relative to unity.

Fatty foods

Corn oil was selected as a possible model simulant. Since corn oil was to model solid foods at 4°C, the sandwich-cell migration data have been plotted in Fig. 9. At each sampling, the cell contents were mixed, yet the amount of migration still correlated well with the square root of time. This fact suggests that diffusional (or any mass transfer) resistance to DOA migration in the corn-oil phase was negligible. Also while DOA is very slightly soluble in aqueous solvents, it is completely miscible with corn oil. Therefore, the partition coefficient for DOA between corn oil and PVC would be expected to be orders of magnitude larger than that with aqueous solvents. These facts lead to the conclusion that, for DOA migrating from PVC to corn oil, the term β in equation (6) is large compared to unity. Equation (6) thus reduces to equation (1). The corn-oil data in Fig. 9 then led to an estimate of $D_p \sim 2{\cdot}3 \times 10^{-12}$ cm^2/sec at 4°C. [This value of D_p is significantly below the value extrapolated from the Quackenbos (1954) data in Fig. 1 (*c.* 2×10^{-10} cm^2/sec). Most probably the difference results from the fact that the Quackenbos PVC contained 60 phr of DOA whereas, in this work, there was a loading of about 32 phr. As discussed in this paper, D_p is a strong function of the plasticizer concentration.]

Next, when we examine the fatty foods studied (beef fat, chicken breast skin, ground and lean beef and fish fillets), we note that in all cases the quantity of DOA migrating into these foods was less than that into corn oil under comparable conditions. We assume that little, if any, of the food penetrated the PVC film since, at 4°C, the mobility of the food oils and carbohydrates must be very small. Then, if equation (6) applies, we have estimates of all parameters except β. Using the migration values after 7 days, β values for the fatty foods were determined as shown in Table 4. Also shown in Table 4 are the percentages of fat in the various materials; β correlates well with the latter figure for all materials except the fish (Fig. 10). The explanation for this relationship between β and the percentage fat is not clear. In the group $\beta = K(D_s/D_p)^{\frac{1}{2}}$, it is unlikely that D_p varies with the type of food unless some food component (related to fat content) can penetrate the PVC film. A more reasonable first approximation is that both K and D_s are functions of the fat content. If it is assumed that these parameters both increase linearly with fat content, then β should be proportional to the fat content raised to the 1·5 power. The experimental data in Fig. 10 show the slope of the correlating line to be 1·51.

Using the value of β for chicken breast skin (0·92), the migration of DOA can be estimated as a function of time using equation (6). The broken line in Fig. 9 is the predicted result. Experimental data for two intermediate times (2 and 4 days) are somewhat higher than predicted but the agreement is reasonable.

For fish fillets, the superficial fat content is very low (*c.* 0·1%) but the migration of DOA into the fish was still significant. It is speculated that the fish fillets may have had an oily surface layer and thus the bulk percentage of fat would not be an appropriate correlating variable. Also, fish oils differ significantly from the

Table 4. *Calculated β values for fatty foods*

Food	M_t*	β†	Fat content (%)
Beef fat	29	2·71	79
Chicken-breast skin	19	0·92	36
Ground beef	12	0·43	22
Fish	2·4	0·064	0·1
Lean beef	1·4	0·036	4·4

*Quantity of DOA (mg/dm^2) migrating in 7 days at 4°C.
†Calculated from equation (6)—$M_t = 2C_{p_0}(D_p t/\pi)^{\frac{1}{2}}[\beta/(1 + \beta)]$—with $C_{p_0} = 0{\cdot}30$ g/cm^3, $D_p = 2{\cdot}3 \times 10^{-12}$ cm^2/sec, and $t = 7$ days $= 6 \times 10^5$ sec.

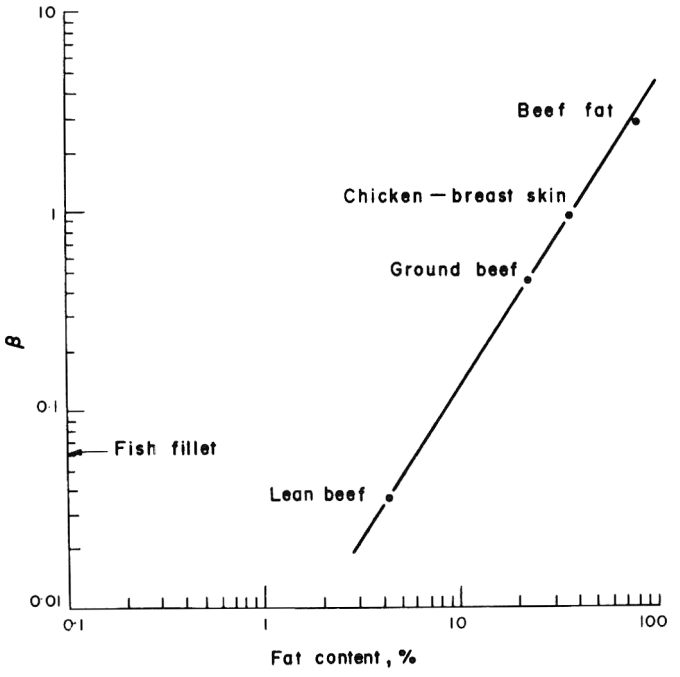

Fig. 10. Correlation of β values for various fatty foods with the percentage fat content.

fats in beef and poultry so that it is likely that K and D_s values for DOA into fish were also different.

The correlation proposed above between β and the percentage of fat may be fortuitous, or there may be an alternative explanation from the one proposed. Our reasoning has implicitly assumed that the diffusion coefficient of DOA in the plasticized PVC remains approximately constant during the extraction experiments. Actually the value of D_p should decrease somewhat as DOA is removed but, also, as corn oil penetrates the PVC, additional plasticizer is added, which should increase D_p. Likewise, for foods, even though less DOA is removed, D_p should drop, but there still appears to be some compensating penetration to keep the *effective* DOA diffusion coefficient nearly constant.

Conclusions

In summary, the few food and solvent data reported in this paper and those from previous investigations do not provide an adequate data base to develop reliable predictive correlations. It does appear reasonable, however, to simulate aqueous solid foods by tests using quiescent water (and, for aqueous liquid foods, stirred water). For fatty solid foods, the use of quiescent corn oil as a simulating solvent would extract more plasticizer than would the food. The corn oil data can be used, however, to estimate a diffusion coefficient of the plasticizer in the PVC film. Then equation (6) is suggested as a correlating equation where the only unknown is the parameter β. For foods of similar fat quality, β appears to be a strong function of the percentage of fat but, at present, there is no way of estimating β *a priori* without some experimental data. Perhaps a direction for future research would be the better characterization of the fatty components of food; this might allow simple experiments to be carried out to estimate the product $KD_s^{\frac{1}{2}}$ so that, with migration data into corn (or other) oil to provide $D_p^{\frac{1}{2}}$, an estimate of β could be made and equation (6) could be used to predict migration of plasticizers between PVC films and foods.

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THE EFFECTS OF NITRILOTRIACETATE ON CATION DISPOSITION AND URINARY TRACT TOXICITY

R. L. ANDERSON, C. L. ALDEN and J. A. MERSKI

The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45247, USA

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Summary—In this review a summary is presented of the experimental evidence that has led to the development of hypotheses to explain how the chronic ingestion of nitrilotriacetate (NTA), which is a non-mutagenic, non-metabolized and non-accumulating compound, might induce urinary tract toxicity that can lead to neoplasia. The hypotheses attribute the toxic process to alterations in divalent cation (M^{2+}) distribution in the urinary tract during the processing of NTA for excretion in the urine. The hypotheses do not identify a 'carcinogen' *per se* but rather define conditions that must exist for the initiation and propagation of toxicity that is an essential precursor of and accompanies tumours associated with chronic, high dosage NTA ingestion. The proposed hypotheses are consistent with all available information on NTA toxicity and define ingestion doses that do not alter urinary tract M^{2+} distributions and do not initiate urinary tract toxicity after chronic exposure. The existence of no-effect doses that must be exceeded for the initiation and propagation of toxicity negates the validity of mathematical extrapolations of possible tumour incidence at doses below the threshold levels from the results obtained in chronic, high dosage experiments. The thresholds determined in the experimental animals are several orders of magnitude greater than human exposure via drinking-water based upon measured NTA concentrations in Canada where NTA has been used in detergents since 1970.

Introduction

Trisodium nitrilotriacetate monohydrate ($Na_3NTA.H_2O$) is a metal sequestering agent that is an effective ingredient for laundry detergents. Reviews of mammalian toxicity and environmental effects of NTA have been published (Foley, Becking, Muller *et al.* 1977; Shapiro, Chapman, Dick, *et al.* 1978; Thayer & Kensler, 1973). Prior to presentation of the hypotheses of how NTA induces urinary tract toxicity, several facts about NTA must be noted.

1. NTA is manufactured as $Na_3NTA.H_2O$ and is more than 99% pure (Monsanto Industrial Chemicals Co.). The impurities are mostly inorganic and probably do not contribute to NTA toxicity.
2. Ingested NTA is readily absorbed from the gut by a zero-order process. There is considerable species variation in the proportion of single oral doses recovered in the urine. Man shows the lowest absorption (12%; Budny & Arnold, 1973) and rodents and dogs the greatest (70–96%; Michael & Wakim, 1971).
3. In all of the species examined it has been found that the only route of excretion of systemic NTA is via the urine as shown by the absence of ingested NTA in the bile (Michael & Wakim, 1971) and the absence of [^{14}C]NTA in the faeces voided during a 72-hr period when [^{14}C]NTA was infused iv (R. L. Anderson, unpublished data 1979). Renal clearance of NTA is by filtration with no evidence for either tubular cell secretion or resorption (A. Licht, N. S. Bricker, R. E. Papendick & R. L. Anderson, unpublished data, 1981).
4. The primary structure of NTA is not altered in passage through several mammalian species including man following acute exposure and through the rat following ingestion to equilibrium (Budny, 1972; Budny & Arnold, 1973; Michael & Wakim, 1971).
5. Only the bone and kidney attain NTA concentrations greater than that in the plasma in equilibrated rats. The relatively high kidney NTA concentrations can be attributed to entrappment of a small volume of urine (*c.* 50 μl/g tissue) with its 130–200-fold greater NTA concentrations than plasma (Anderson, 1980). The finding of NTA in bone is expected since chemicals that form complexes with divalent cations are known to concentrate in bone (Shtacher & Anbar, 1966).
6. Three distinct pools of NTA can be distinguished in the blood—a blood cells pool, a plasma-protein (mol wt >10,000) pool and a plasma ultrafiltrate (mol wt <10,000) pool. The concentration of NTA in each of these pools is a linear function of the dietary level of NTA, at least in the rat (R. L. Anderson, unpublished data, 1980).
7. Absorption, blood and organ distribution, and excretion of NTA all demonstrate zero-order kinetics (Anderson, 1980).
8. Subchronic (Anderson & Kanerva, 1979; Mahaffey & Goyer, 1972; Nixon, 1971) and chronic (Table 1) exposure studies show that high NTA intake is associated with urinary tract toxicity and an increased incidence of urinary tract neoplasms. There is no consistent evidence that sites other than the urinary tract show any toxicity (including tumours) even after chronic ingestion of NTA at very high doses (up to 79 μmol/g diet). In this

Table 1. *Summary of chronic exposure studies with nitrilotriacetic acid (NTA)*

NTA form	Method of administration	Duration of treatment	Dose (% in DW or diet)	Urinary tract neoplasms (no. of animals affected/ total no. of animals)		Reference*
				Renal tubular cell neoplasms	Bladder transitional cell neoplasms	
Studies in rats						
Na_2NTA	DW	84 wk (20 ml DW/day, 5 days/wk)	0	0/30	0/30	1
			0·5	1/30	0/30	
			0·5†	2/30	0/30	
$Na_3NTA.H_2O$	DW	704 days	0	5/186	0/186	2
			0·1	29/183	0/183	
$Na_3NTA.H_2O$	Diet	24 months (interim kill at 19 months)	0	0/71	0/71	3
			0·03	0/37	0/37	
			0·15	0/31	0/31	
			0·50	1/31	0/31	
CaNaNTA	Diet		0·50	2/38	0/38	
$Na_3NTA.H_2O$	Diet	24 months	0	0/24	0/24	4
			0·02	0/48	0/48	
			0·2	0/48	1/48‡	
			2·0	8/48	6/48	
$Na_3NTA.H_2O$	Diet	18 months + 6 months untreated	0	0/80	0/80	4
			0·75	1/100	4/100	
			1·50	2/100	2/100	
H_3NTA	Diet	18 months + 6 months untreated	0·75	1/100	2/100	4
			1·50	7/100	12/100	
Studies in mice						
Na_2NTA	DW	26 wk (5 ml/day, 5 days/wk)	0	1/76	1/76	5
			0·5	1/74	0/74	
			0·5§	0/76	0/76	
$Na_3NTA.H_2O$	Diet	18 months + 3 months untreated	0	0/80	0/80	4
			0·25	0/100	0/100	
			0·50	0/100	0/100	
H_3NTA	Diet	18 months + 3 months untreated	0·75	5/100	0/100	4
			1·50	28/100	0/100	

DW = Drinking-water

*References: (1) Lijinsky, Greenblatt & Kommineni, 1973; (2) Goyer, Falk, Hogan, Friedman & Richter, 1981; (3) Nixon, Buehler & Niewenhuis, 1972; (4) NCI, 1977; (5) Greenblatt & Lijinsky, 1974.

†In combination with 0·2% $NaNO_2$.

‡A papilloma.

§In combination with 0·1% $NaNO_2$.

regard it is important to note that the urinary tract is the only tissue that is unquestionably exposed to a medium (urine) containing NTA that is not present as a divalent metal complex, i.e. at all sites other than the urine M^{2+}:NTA (M:M) = >1 (Fig. 1).

9. NTA is neither a mutagen nor a teratogen as attested by its essentially negative response in several assay systems (Foley *et al.* 1977).

Morphogenesis of NTA-associated tubular cell tumours

Based on structural changes observed in the renal tissue of rats ingesting high levels of NTA, it is possible to describe two sequences of morphological alterations that strongly suggest a relationship between the nephrotoxic and tumorigenic actions of NTA (Fig. 2). One sequence (Fig. 2a) begins with the development of vacuoles of various sizes in the cytoplasm of the proximal convoluted tubular (PCT) epithelial cells in the renal cortex of rats that are treated with NTA (Merski, 1981). Under the light microscope these lesions appear in adjacent groups of tubule cross section which presumably represent different levels of a single nephron. Distribution of altered nephrons in the cortex is multifocal. Electron microscopic examination of affected tubules and comparison with previous descriptions of cytoplasmic vacuolization (Maunsbach, 1969) demonstrates that the vacuoles result from swelling of the endocytotic/lysosomal system in PCT cells (Plate I).

Acute gavage studies as well as subchronic and chronic feeding studies show that the vacuolar response is dose dependent and *c.* 0·073 mmol/kg/day does not induce this effect. As the dose of NTA is increased above 0·073 mmol/kg both the size and

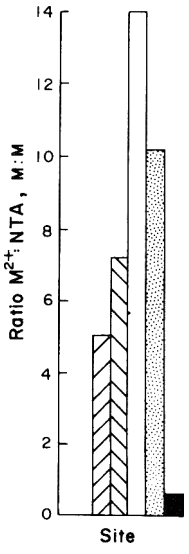

Fig. 1. Ratio (M:M) of M^{2+} (Ca + Mg + Zn) to NTA in the diet (▨) and in the faeces (▧), plasma (□), plasma ultrafiltrate (mol wt < 10,000; ▨) and urine (■) of rats ingesting NTA at a dose of 73 μmol/g diet.

number of vacuoles in an affected tubule and the number of affected tubules increase. Time-course experiments have demonstrated that the vacuoles develop within 1·5–6 hr and in the most extreme instances persist for up to 72 hr after a single dose of 7·3 mmol NTA/kg body weight given by gavage (Merski, 1981). In longer term studies vacuolization persists as long as NTA administration continues in high doses (Alden & Kanerva, 1980; Alden, Kanerva, Anderson & Adkins, 1981; Nixon, Buehler & Niewenhuis, 1972) but is reversible if NTA ingestion is stopped (M. C. Myers, R. L. Kanerva, C. L. Alden & R. L. Anderson, unpublished data, 1981).

With continuation of the NTA-associated insult by daily gavage or feeding, the vacuolated proximal convoluted tubular cells frequently develop hypertrophy/hyperplasia (Plate II). Hyperplasia of the vacuolated PCT epithelium occurs consistently in rats fed 2% $Na_3NTA.H_2O$ for 4 wk or longer (Alden & Kanerva, 1980; Alden *et al.* 1981). In addition, occasional foci of exaggerated hyperplasia, termed epithelial tubular hyperplastic nodules, develop in the vacuolated PCT cells (Plate III). These lesions are reversible if NTA ingestion is stopped (Myers *et al.* unpublished data, 1981). The sequential development of these lesions through adenomatous hyperplasia (Plate IVa) to tubular neoplasia (Plate IVb) was evident in a 2-yr feeding study at a level of 2% $Na_3NTA.H_2O$ in the diet (Alden & Kanerva, 1980; NCI, 1977). The sequence in Fig. 2a can proceed to intermediate stages (vacuoles plus nodular hyperplasia) without tumour development even after 24 months of exposure (Nixon *et al.* 1972).

The second sequence of structural alterations (Fig. 2b) involves an increase in the incidence of nonspecific injury in the renal cortex of rats subchronically treated with high doses of NTA (Alden & Kanerva, 1980; Alden *et al.* 1981). These nonspecific lesions included tubular atrophy, glomerular fibrosis, and interstitial lymphofibrocytic response and result in a directly proportional increase in nonvacuolated tubular cell hyperplasia (Plate V). These lesions represent a significant exacerbation of the structural alterations normally associated with age-related nephrosis of rats. Age-related nephrosis occurs spontaneously in the strains of rats that have been used in chronic NTA experimentation. Loss of parenchyma in severe age-related nephrosis has been previously associated with a tubular regenerative response including nonvacuolated hyperplastic PCT cells with basophilic or, less commonly, eosinophilic cytoplasm (Cohen, Anver, Ringler & Adelman, 1978). A higher incidence of renal tubular tumours has been noted in

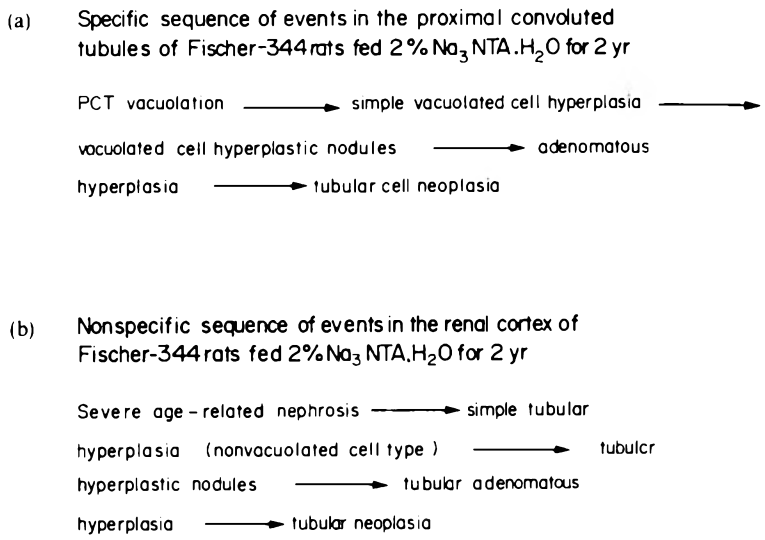

Fig. 2. Sequence of renal tubular cell toxicity observed in rats exposed to high doses of NTA. (a) Sequence associated with cytoplasmic vacuoles in proximal convoluted tubule epithelium. (b) Exacerbation of nonspecific, age-related nephrosis. (See text for further details.)

strains of rats with a high incidence of severe age-related nephrosis in lifetime studies compared to 2-yr studies, suggesting a relationship between renal tumour formation and ageing changes in the kidney (Cohen *et al.* 1978).

Hence, on the basis of observed structural changes, two morphogenic pathways for renal tubular tumour formation are apparent in rats fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr. One pathway is associated with a specific indicator of NTA-associated toxicity, vacuolization. The second pathway is associated with nonspecific toxicity and is manifested as an exacerbation of age-related nephrosis. Based on the type and severity of PCT hyperplasia observed, both these pathways appear to make a quantitatively similar contribution to the development of NTA-associated renal tumours.

No changes in renal tubular structure detectable under the light microscope are observed in rats given either a single dose of 0·073 mmol NTA/kg body weight by gavage (Merski, 1981) or diets containing 0·02% NTA (0·73 μmol/g diet; NCI, 1977) or 0·03% NTA (1·1 μmol/g diet) (Nixon *et al.* 1972) for 2 yr. Doses of NTA that do not result in morphological evidence of the specific nephron toxicity, whether in acute, subchronic or chronic feeding studies, do not induce tubular neoplasms in chronic studies and are 'no (morphological)-effect doses'.

Specific relationship between tubular cell zinc resorption and tubular cell toxicity

A detailed discussion of the relationship between increased renal tubular zinc resorption and tubular cell toxicity in rats exposed to NTA has been published (Anderson, 1981). This work indicated that each stage in the toxicity sequences shown in Fig. 2 is dependent upon NTA attaining sufficiently high levels in the plasma ultrafiltrate (UF) to cause an increase in the plasma UF concentration of zinc (UF_{Zn}). During renal clearance of the plasma UF the majority of the increased UF_{Zn} but not the UF_{NTA} is resorbed, probably by the PCTs. This increased zinc resorption by the PCT results in an increase in the renal tissue zinc concentration greater than that of the tissue NTA concentration. This site-specific increase in tissue zinc has been demonstrated to be a necessary condition for both the initiation and progression of the NTA-associated renal tubular toxicity which precedes and accompanies neoplasias at this site.

Dose-response studies show that the plasma UF_{NTA} must exceed a concentration of 20 μM before it causes an increase in plasma UF_{Zn} (Anderson, 1981). Once this threshold of 20 μM for NTA in the plasma UF is exceeded then both UF_{Zn} and PCT toxicity increase in proportion to the level of NTA in the diet and the UF. If dietary zinc is made limiting then the extent of PCT toxicity induced by a constant high urinary load of NTA is markedly reduced.

Since renal clearance of NTA at nephrotoxic doses increases not only renal resorption of zinc but also increases urinary excretion of zinc, increased urinary levels of zinc can also define when the plasma UF_{NTA} threshold has been exceeded. For example, Nixon *et al.* (1972) noted that 2 yr of ingesting a diet containing 0·03% $Na_3NTA.H_2O$ caused no change in urinary zinc and it produced no nephrotoxicity. In contrast, 0·15% $Na_3NTA.H_2O$ and 0·5% $Na_3NTA.H_2O$ or 0·5% CaNaNTA in the diet produced a dose-dependent increase in urinary zinc and nephrotoxicity. Subchronic feeding studies with NTA in the same dose range (0, 0·02, 0·2 and 2·0% $Na_3NTA.H_2O$ and H_3NTA) showed that only the highest dose caused increased urinary zinc (R. L. Anderson, unpublished data, 1980) and the 2-yr bioassay showed that only the highest dose was associated with renal tubular cell tumours (NCI, 1977).

It was recently reported in a National Institute of Environmental Health Sciences study that 0·1% $Na_3NTA.H_2O$ in the drinking-water results in an increased incidence of tubular cell neoplasms (Goyer, Falk, Hogan, Friedman & Richter, 1981), so the effect of this treatment on urinary Zn was ascertained. Rats consume about 2 ml of water for each gram of diet (Table 2), so 0·1% $Na_3NTA.H_2O$ in drinking-water is equivalent to approximately 0·2% in the diet, a dose greater than that already known to increase urinary Zn (Nixon *et al.* 1972). Table 2 also shows that consumption of water containing 0·1% $Na_3NTA.H_2O$ resulted in a six-fold increase in urinary Zn, so that the toxicity associated with this NTA ingestion level is consistent with a relationship between tubular cell toxicity and altered Zn metabolism.

These findings have led to the formation of a hypothesis that NTA-associated renal tubular tumour formation is an epigenetic process which occurs as the final step in a sequence of pathological changes in the renal tubular epithelium. This sequence of events occurs as a direct result of changes in zinc metabolism which only develop when the UF_{NTA} attains or exceeds a level of 20 μM on a chronic basis. This hypothesis is not to be construed as implying that Zn is a renal tubular cell 'carcinogen' but it does imply that increased UF_{Zn}, PCT Zn resorption, tissue Zn accumulation and urinary Zn excretion are 'necessary conditions' for initiation of the PCT damage that is an essential prerequisite for neoplasia development during lifetime exposure to high levels of NTA. A direct consequence of this hypothesis is that it negates the appropriateness of any mathematical extrapolation of possible toxicity, including tumours, at NTA exposure levels that clearly do not attain this threshold from results obtained at doses that exceed the described threshold ($UF_{NTA} \geqslant 20$ μM/ml).

In order to place in perspective the lowest ingested dose of NTA that was associated with increased renal tubular cell neoplasms (0·1% $Na_3NTA.H_2O$ in drinking-water; Goyer *et al.* 1981), the exposure level at this dose has been compared to human exposure at the mean NTA concentration in drinking-water in Canada where NTA has been used in laundry detergents (Malaiyandi, Williams & O'Grady, 1979). The comparative exposure data are summarized in Table 3. These show that 0·1% $Na_3NTA.H_2O$ in drinking-water is a 1,000,000-fold exaggeration of human ingestion of NTA from Canadian drinking-water while the systemic load (NTA consumed × % absorbed) in the rats is about 6,000,000 times that which would result in humans exposed to Canadian drinking-water.

In summary, it has been demonstrated in a variety of studies that initiation of renal tubular cell toxicity associated with NTA ingestion depends upon NTA

Table 2. *Effect of 0·1% $Na_3NTA.H_2O$ in drinking-water on male Charles River rats during 3 wk of* ad lib. *intake*

Parameter	Drinking solution	
	Distilled water	0·1% $Na_3NTA.H_2O$
Weight gain	179 ± 15	160 ± 8
Water intake/g diet consumed	1·9 ± 0·1	1·9 ± 0·2
H_2O consumption (ml/kg body weight)	144 ± 6	141 ± 14
Urine: volume (ml/kg body weight)	84 ± 4	85 ± 13
pH	7·4 ± 0·1	7·9 ± 0·2
Ca (μmol/g diet consumed)	3·2 ± 0·8	2·9 ± 0·6
K (μmol/g diet consumed)	194 ± 10	205 ± 22
Mg (μmol/g diet consumed)	8·2 ± 1·4	6·1 ± 0·6
Na (μmol/g diet consumed)	144 ± 6	172 ± 8
Zn (nmol/g diet consumed)	10 ± 1	66 ± 4

Young adult rats (200 g) were given laboratory chow (Ralston Purina Co., Inc.) and either distilled water or distilled water containing 1 g $Na_3NTA.H_2O$/litre. Feed and drinking solution were offered *ad lib.* for 21 days. Feed and water consumption were measured continuously.

attaining sufficiently high concentrations ($\geqslant 20\ \mu M$) in the plasma UF so that it causes an increase in the Zn content of this pool probably through formation of a ZnNTA complex. After the UF passes the glomerulus, the proximal tubules resorb a majority of the increased Zn but not the NTA. The initial response noted is the presence of vacuoles in the tubular cells reflecting a response of the endocytotic/lysosomal system. If the NTA dosing is continued the vacuolated PCTs tend to become hyperplastic and nonspecific tubular cell injury is exacerbated. Each of these steps is dependent on the availability of Zn in the plasma; at constant NTA levels, lowering Zn availability reduces the injury and increasing Zn availability exacerbates it.

Finally, if a sufficient dose of NTA is provided on a chronic basis tubular cell neoplasms can develop. In contrast, doses of NTA of *c.* 1 μmol/g diet or less do not yield UF_{NTA} concentrations sufficiently high ($\leqslant 20\ \mu M$) to elevate the UF_{Zn} and thus do not provide the 'necessary condition' to initiate the toxic process and, therefore, are 'no-effect' doses. The 'no-effect' dose established in rats (*c.* 1 μmol/g diet or 50 μmol/kg body weight/day) is approximately a 100,000-fold exaggeration of the mean human exposure level from drinking-water in Canada where NTA has been used in detergents since 1970.

Urinary tract transitional cell neoplasms associated with chronic exposure to NTA

Of the chronic exposure studies listed in Table 1, only the NCI bioassay with Fischer-344 rats reported an NTA-associated incidence of urinary tract transitional cell neoplasms. A detailed discussion of these data has been presented elsewhere (Anderson, 1979).

Proposed mechanism for transitional cell carcinoma development in rats ingesting NTA

The earlier portions of this review described the fate of systemic NTA and the fact that the only route of excretion is the urine. As the dose of NTA in the diet is increased the daily load of NTA voided in the urine increases and when dietary NTA is c. 40 μmol/g diet (0·76% H_3NTA) the urine contains as much NTA as it does total M^{2+} (sum of $Ca^{2+} + Mg^{2+} + Zn^{2+}$) on a molar basis. Greater urinary loads of NTA do

Table 3. *Comparison of NTA exposure in rats in a chronic drinking-water exposure study with the mean consumption by humans from drinking-water in Canada*

Parameter	Rats*	Humans* (Canadian drinking-water)	Rats: Humans
NTA concentration	1 mg $Na_3NTA.H_2O$/ml (1)	$2·82 \times 10^{-6}$ mg NTA/ml (2)	$3·5 \times 10^5$
H_2O consumption (ml/kg body weight/day)	139 ml/kg/day (3)	35 ml/kg/day (3)	3·97
NTA consumption (mmol/kg/day)†	0·51	$0·52 \times 10^{-6}$	10^6
Absorption (% of ingested dose)	70 (4)	12 (5)	5·8
Systemic load (mmol/kg body weight/day)‡	0·36	$0·06 \times 10^{-6}$	$6·0 \times 10^6$

*References are given in brackets: (1) Goyer, Falk, Hogan, Friedman & Richter (1981); (2) Malaiyandi, Williams & O'Grady (1979); (3) Altman & Dittmer, 1968; (4) Michael & Wakim (1971); (5) Budny & Arnold (1973).
†Mol wt $Na_3NTA.H_2O$ = 275; H_3NTA (mol wt = 191) was used to determine Canadian values.
‡NTA consumed × % absorbed.

not result in greater amounts of urinary M^{2+} excretion and thus at >40 μmol/g diet the urine must contain uncomplexed NTA. The presence of uncomplexed NTA in the urine results in the extraction of M^{2+} from the urinary tract epithelial tissue. When the rate of M^{2+} extraction exceeds the rate at which M^{2+} can be replaced by diffusion from the blood there is a specific loss of Ca from the bladder tissue. The Ca lost from the tissue probably represents Ca from the accessible intercellular pool. Organ culture studies with bladder tissue have shown that a medium low in Ca results in hyperplasia and endophytic growth of the epithelium and in the absence of Ca in the medium there was extensive epithelial cell erosion, probably through desmosome disruption (Reese & Friedman, 1978). It is proposed that ingestion of high doses of NTA results in the voiding of urine containing uncomplexed NTA which induces bladder toxicity via extraction of the tissue intercellular Ca pool, which disrupts cell–cell junctions and provides a mitogenic stimulus.

NTA-associated pathology of urinary tract transitional epithelium

Short-term feeding of NTA to rats is associated with a rapid development of significant clinical and morphological evidence of urinary tract toxicity (Alden *et al.* 1981; Anderson, 1979; Anderson & Kanerva, 1978a,b). Polyuria, alkalinuria, and haematuria develop rapidly and consistently in male Charles River (Sprague–Dawley-derived) rats fed 2% $Na_3NTA.H_2O$ for 28 days (Anderson, 1979; Anderson & Kanerva, 1978b). Morphological alterations in these rats include hydronephrosis and ureteral dilation (Plate VI). Microscopically, transitional epithelium of the renal pelvis becomes eroded and/or ulcerated and hyperplasia occurs at the periphery of ulcers and in erosive foci (Plate VII). The transitional epithelium of the ureters is similarly affected. When examined under the light microscope the bladder epithelium appears unaffected in Charles River (Sprague–Dawley-derived) rats subchronically fed 2% $Na_3NTA.H_2O$. However, recent results of scanning electron microscopy of the transitional epithelium of the bladders of rats fed 1·5% H_3NTA in a subchronic study demonstrate that erosive alterations are present even though under a light microscope the bladders appear normal (M. Myers, personal communication, 1981).

Mild hydronephrosis and transitional cell hyperplasia occurred in male Fischer-344 rats fed 2% $Na_3NTA.H_2O$ for 24 months in the NCI Bioassay (Plate VIII). In addition, transitional epithelial tumours occurred in the renal pelvis, ureter and urinary bladder (Plate IX). However, erosion and ulceration of transitional epithelium was not evident after 24 months of feeding NTA. The occurrence of erosion and ulceration in Charles River (Sprague–Dawley-derived) rats given NTA subchronically compared to the occurrence of hyperplasia with an absence of erosion and ulceration after chronic administration to Fischer-344 rats is attributable to NTA exposure differences since Charles River (Sprague–Dawley-derived) rats consume about 1·7 times as much feed as Fischer-344 rats (Anderson & Kanerva, 1979). Hence a continuum of events is apparent in the urothelium of rats chronically fed high doses of NTA, beginning as a toxicological response (erosion) leading to proliferation (hyperplasia), and after chronic exposure progressing occasionally to neoplasia. 'No-effect' doses of NTA on the urothelium have been demonstrated in two studies (NCI, 1977; Nixon *et al.* 1972) in which 174 male and female rats ingested a diet containing up to 0·5% $Na_3NTA.H_2O$ or H_3NTA for 18–24 months.

Effects of dietary NTA dose on urinary calcium excretion

Ingestion of diets containing up to 18 μmol NTA/g (0·5% as $Na_3NTA.H_2O$) do not alter the urinary Ca levels relative to values observed in control animals when measured in subchronic (Anderson & Kanerva, 1978b) and chronic (Nixon *et al.* 1972) feeding studies. When the dietary NTA level is >27 μmol/g diet, there results a dose-dependent increase in urinary Ca and the collected urine contains grossly visible quantities of crystalline CaNaNTA (Anderson & Kanerva, 1978b). A comparison of the effects of NTA dietary concentration on the amount of insoluble NTA in the voided urine (determined in subchronic feeding studies) and the incidence of bladder total neoplasms [papillomas and carcinoma (NCI, 1977)] showed a very marked similarity suggesting a relationship between these two phenomena (Anderson, 1980).

Four observations suggested a rationale for the relationship between the effects of NTA on urinary Ca excretion and the development of bladder neoplasms in chronic feeding studies. (1) It has been known for many years that tumorous tissue has much lower Ca concentrations than normal tissue of the same origin (Coman, 1953). (2) Published reports have associated low extracellular Ca with breakdown of cell–cell attachments (Hennings, Michael, Cheng, *et al.* 1980; Reese & Friedman, 1978). Loss of cell adhesiveness could theoretically lead to premature sloughing and subsequently to erosion and ulceration. (3) Bladder

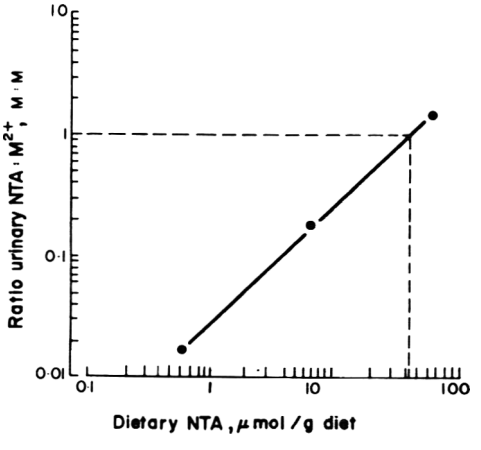

Fig. 3. Ratio (M:M) of urinary NTA to urinary M^{2+} (sum of Ca + Mg + Zn) as a function of dietary NTA concentration.

tissue cultured in a medium low in Ca results in tissue hyperplasia and endophytic growth of the epithelium relative to tissue in the same medium with a higher Ca level (Reese & Friedman, 1978). (4) Alterations of the urinary transitional urothelium were noted only at dietary levels of NTA which resulted in urines containing more NTA than M^{2+} (Fig. 3)—a condition where urinary Ca^{2+} activity would be very low.

Measurement of Ca^{2+} activity (with a Ca specific electrode, Orion calcium electrode model 93-20, Orion Research, Inc., Cambridge, MA) in urine obtained from rats fed doses of NTA which produce NTA:M^{2+} > 1·0 confirmed that such urines contain no more free Ca^{2+} than distilled water even though these urines contain more total Ca than urines from control animals. In contrast, urines from control animals had free Ca^{2+} activities of *c.* $1{\cdot}2 \times 10^{-4}$ M which were readily lowered by addition of NTA *in vitro*. Addition of $CaCl_2$ to urine showing zero Ca^{2+} activity (from rats fed NTA) showed a dose-dependent increase in the electrode response when the added Ca exceeded the excess NTA level in the urine.

The hypothesis that the presence of uncomplexed NTA in the urine could reduce bladder Ca levels was tested by measuring the bladder-tissue cation concentrations in samples from rats fed a dose of NTA (1·5% H_3NTA) that results in urine containing uncomplexed NTA and was associated with bladder carcinoma in a chronic exposure study (Table 1). Figure 4 shows that 4 wk of ingestion of a diet containing 1·5% H_3NTA (79 μmol/g diet) was associated with a 50% reduction in the tissue concentration of Ca but with little change in the tissue Mg, Zn, Na or K content. Thus excretion of urines with uncomplexed NTA can result in a specific reduction in bladder Ca content while it does not alter the tissue content of Zn, a metal with which NTA forms a more stable complex (Cilley & Nicholson, 1971). Thus the Ca pool that was reduced must be more accessible (intercellular) than the Zn pool (intracellular). Bladder tissue from animals fed 1·5% H_3NTA for 4 wk showed no abnormal morphology at the light microscopic level but examination by scanning electron microscopy (SEM) showed that the bladders from four of five rats that ingested this dose of NTA had areas of superficial epithelial desquamation (M. Myers, personal communication, 1981).

The effect of direct bladder infusion of NTA was investigated for its effect on tissue and voided urine M^{2+}. Two doses of NTA were infused (200 and 400 μmol/rat/day) at a rate of *c.* 10 μl/min for 3 days and for 5 days. Control rats were infused with saline. The doses of NTA infused were selected so that the lower dose would provide NTA at a level equivalent to the M^{2+} level in the urine voided by control animals and would thus provide a M^{2+}:NTA (M:M) in the urine of *c.* 1. The higher dose was selected to provide a urine with NTA > M^{2+} and to thus mimic the condition associated with reduced bladder Ca levels noted in feeding studies. The ratios (M:M) of M^{2+} (sum of Ca + Mg) to NTA (determined by isotope dilution) during 72 hr of direct infusion were 1·20 and 0·66 at the low and high doses, respectively. Figure 5 shows the result of 72 hr of infusion of NTA into the bladder on the relative (percentage of values obtained for samples from rats infused with saline) bladder-tissue and voided-urine M^{2+} levels. Of the three M^{2+} species (Ca, Mg, Zn) examined only Ca showed a dose-dependent increase in urinary output with NTA infusion dose. The increase in urinary Ca at the lower NTA infusion rate was not accompanied by a decrease in bladder-tissue Ca but at the higher NTA infusion rate the tissue Ca was decreased. In contrast, urinary Zn was increased to approximately the same extent by the two doses of NTA but bladder-tissue Zn remained fairly constant and certainly 400 μmol NTA/day produced no more of an effect on bladder-tissue Zn than 200 μmol NTA/day. The Mg concentration in the tissue decreased with NTA infusion but this tissue change was not reflected by an increase in urinary Mg. When the infusion at 400 μmol/day was continued for 120 hr, the voided urines contained blood and, therefore, measurements

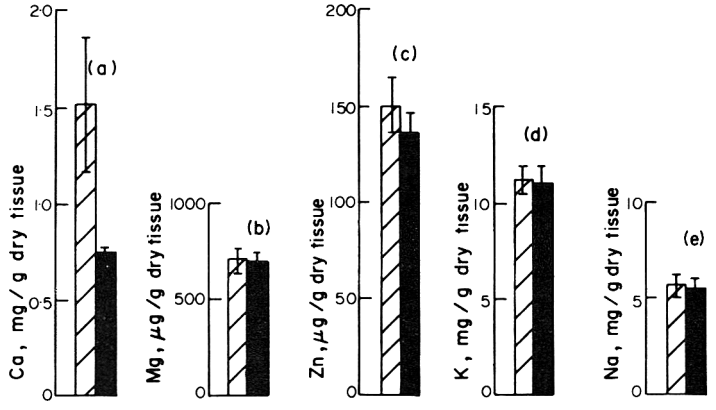

Fig. 4. Bladder tissue concentrations of (a) calcium, (b) magnesium, (c) zinc, (d) potassium and (e) sodium in control female rats (▨) and female rats fed 1·5% H_3NTA in the diet for 4 wk (■). Each value is the mean for five samples and range bars indicate the SEM.

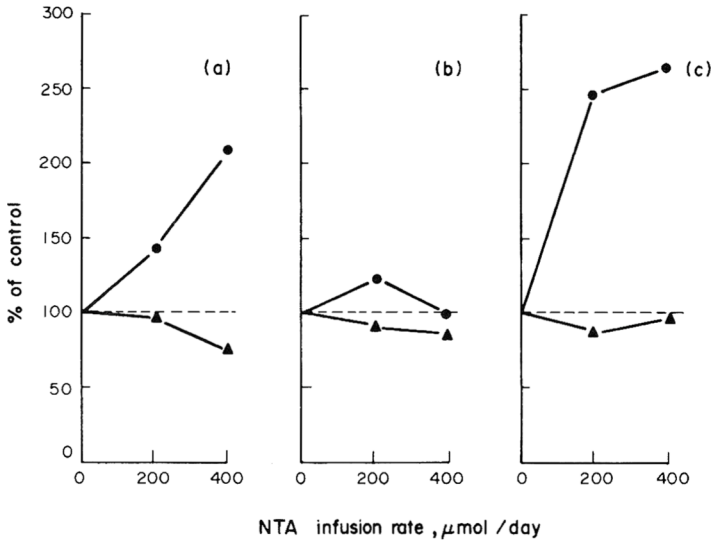

Fig. 5. Urinary excretion (●) and bladder dry tissue concentrations (▲) of (a) calcium, (b) magnesium and (c) zinc in rats the bladders of which were directly infused with Na_2NTA (pH 6·5) for 72 hr at a rate of 10·5 ml/24 hr. Values are expressed as a percentage of the control values that were obtained from rats infused for 72 hr at the same rate with 0·9% saline (pH 6·5). Each treatment was applied to three rats.

of urinary cations are of doubtful value following this treatment.

To test further the hypothesis that uncomplexed NTA in the urine is an essential state for induction of bladder toxicity, the effect on tissue morphology of NTA infused directly into the bladder for 96 hr was ascertained.

The study compared the effects of saline with that of two doses (200 and 400 μmol/day) of Na_2NTA (pH 6·5) and of the higher dose as the $CaNH_4NTA$ complex. Two rats were used for each treatment. A neutrophil response was present in all tissues adjacent to the site of entry of the catheter. The severity of this response was greater in tissues from rats infused with 400 μmol Na_2NTA/day than in those from rats given the same dose of NTA as the Ca complex; the saline-infused tissue showed the least severe response. The tissue near the site of catheter entry was eroded and ulcerated in both bladders exposed to 400 μmol Na_2NTA/day, in one bladder exposed to 200 μmol Na_2NTA/day and in neither bladder exposed to 400 μmol $CaNH_4NTA$. The evaluation of bladder tissue at sites distant from the point of catheter entry were more informative. The bladders from saline-infused rats showed no lesions. The low-dose Na_2NTA and the $CaNH_4NTA$ infusion resulted in a mild to moderate neutrophil response and some epithelial thickening. The high-dose Na_2NTA treatment, in contrast, resulted in a severe neutrophil response, epithelial ulceration/erosion and sites of hyperplasia with appreciable numbers of mitotic figures at sites distant from catheter entry (Plate X).

This work shows several significant facts.

1. Bladder tissue and its associated blood supplies can provide a significant portion of the increased urinary Ca and Zn noted in NTA feeding studies. This suggests that in high-dose NTA feeding studies at least part of the increased Ca and Zn in the urine could be derived from the lower urinary tract and need not be totally derived from decreased tubular cell resorption of these cations.
2. The presence of uncomplexed NTA in the urine can extract Ca from bladder tissue faster than it can diffuse into the tissue from the circulating blood, suggesting that this is the mechanism for the reduced tissue Ca levels noted in the feeding studies. In contrast, the presence of uncomplexed NTA in the urine can extract Zn from the circulation but not from bladder tissue.
3. Even short-term (72 hr) exposure of bladder tissue to urine with uncomplexed NTA (NTA > M^{2+}) reduces bladder-tissue Ca content. In contrast, when the urine contains sufficient M^{2+} to complex the NTA present there is no reduction in bladder-wall Ca concentration.
4. When infusion of a high dose of NTA, which results in urine with uncomplexed NTA, is continued for more than 72 hr the voided urine contains blood, suggesting severe bladder damage. Blood-containing urines have been observed in rats fed diets containing 1·5% H_3NTA, a treatment that does not cause renal pelvis damage, suggesting that the bladder may be the source of this blood (Anderson & Kanerva, 1979).
5. Infusion of doses of NTA resulting in urine with NTA:M^{2+} > 1 does induce lesions of the bladder epithelium. This effect is less severe at an infusion dose that results in a urinary NTA:M^{2+} < 1 either by lowering the NTA dose infused or by infusion of the higher dose as the preformed $CaNH_4NTA$ complex.

These associations between high-dose NTA effects on urinary and bladder tissue Ca distributions have led to the hypothesis that NTA-associated tran-

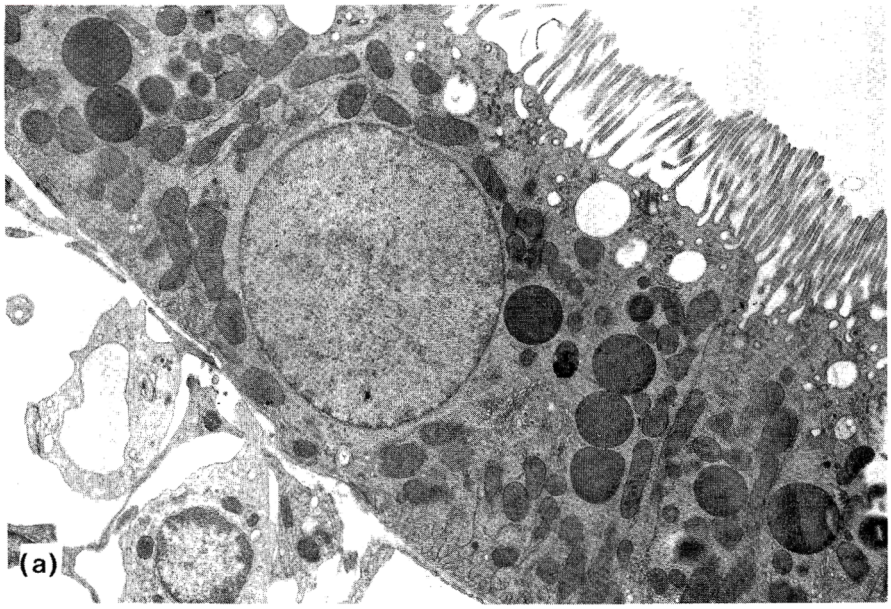

Plate Ia. Portion of a renal proximal tubule from an untreated rat. Uranyl acetate, lead citrate stain × 9600.

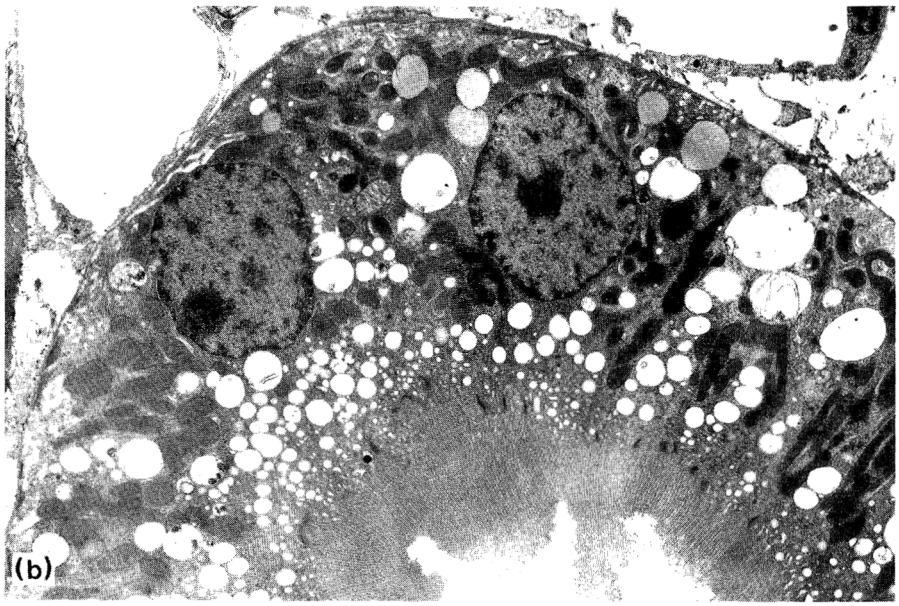

Plate Ib. Increase in the size and number of vacuoles in a proximal convoluted tubule of a rat that was given a 7·3-mmol/kg dose of NTA 6 hr before it was killed. Uranyl acetate, lead citrate stain × 7800.

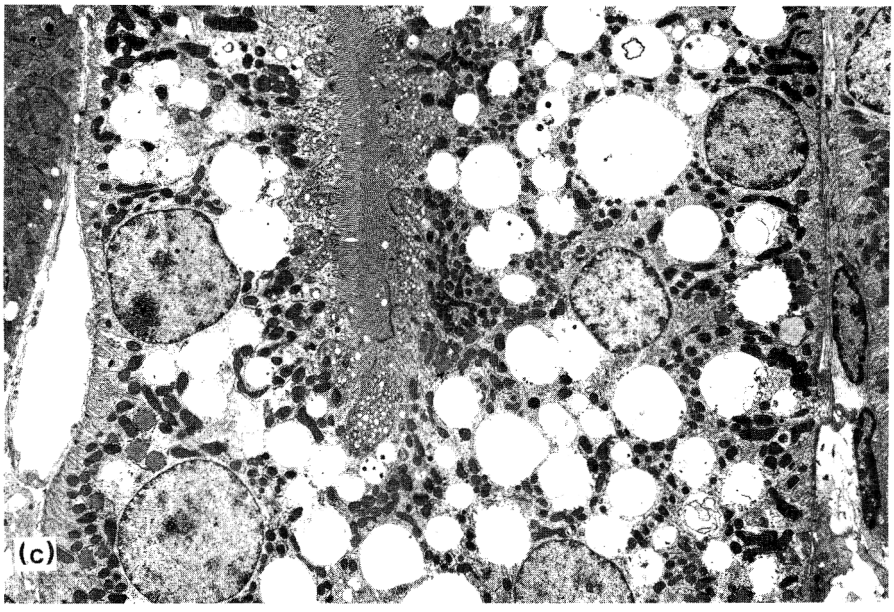

Plate Ic. Highly vacuolated portion of a proximal convoluted tubule from a rat that was given a 7·3-mmol/kg dose of NTA 6 hr before it was killed. Uranyl acetate, lead citrate stain. × 7200.

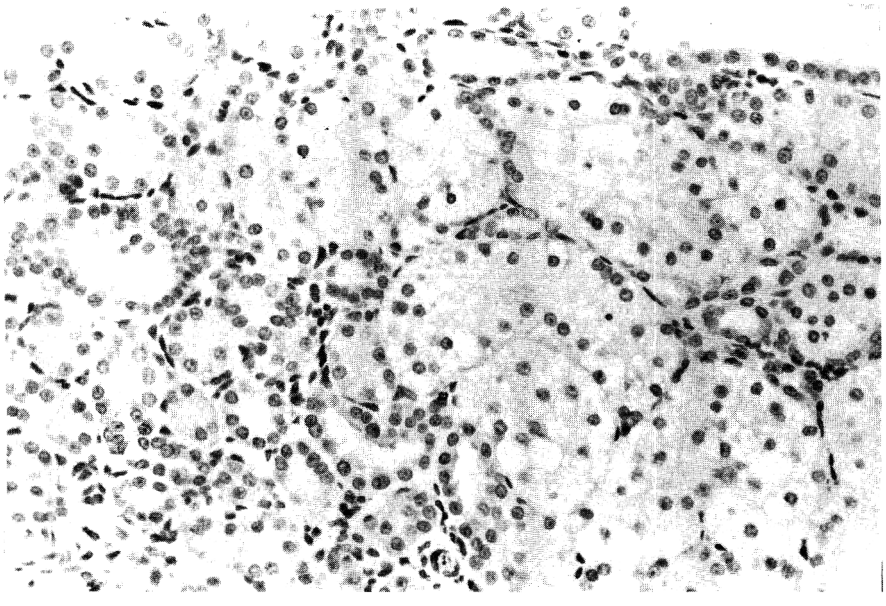

Plate II. Photomicrograph of a section of a kidney of a Fischer-344 rat fed 2% $Na_3NTA.H_2O$ for 2 yr showing a focus (nephron) of proximal convoluted tubular epithelium undergoing simple hyperplasia comprised of cells with vacuolated cytoplasm, a specific NTA effect. × 145.

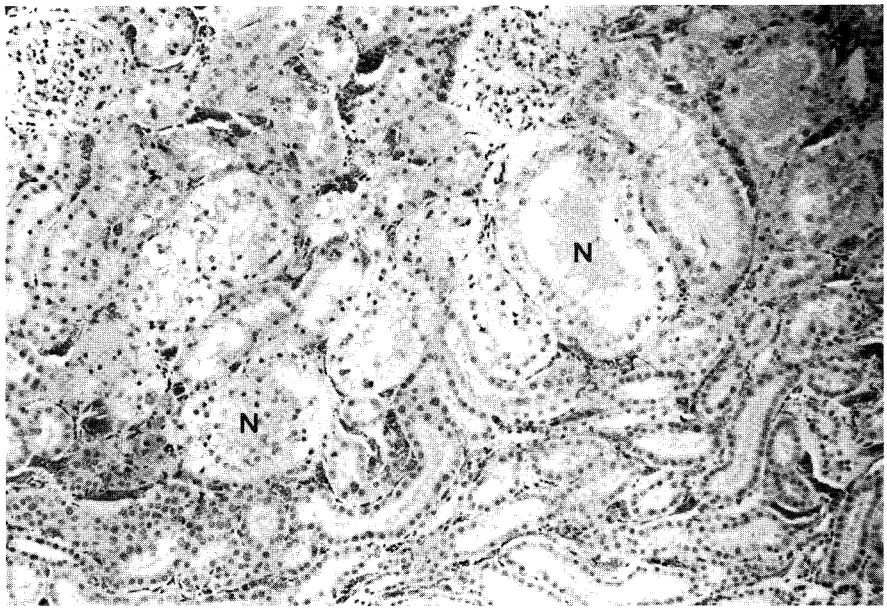

Plate III. Photomicrograph of a portion of a kidney from a male Charles River (Sprague–Dawley-derived) rat fed $Na_3NTA.H_2O$ at 2% in the diet for 42 days showing a focus (single nephron?) of vacuolated-cell-type hyperplasia. A transition from simple hyperplasia to hyperplastic tubular nodules (N) is apparent in this single focus. × 72.

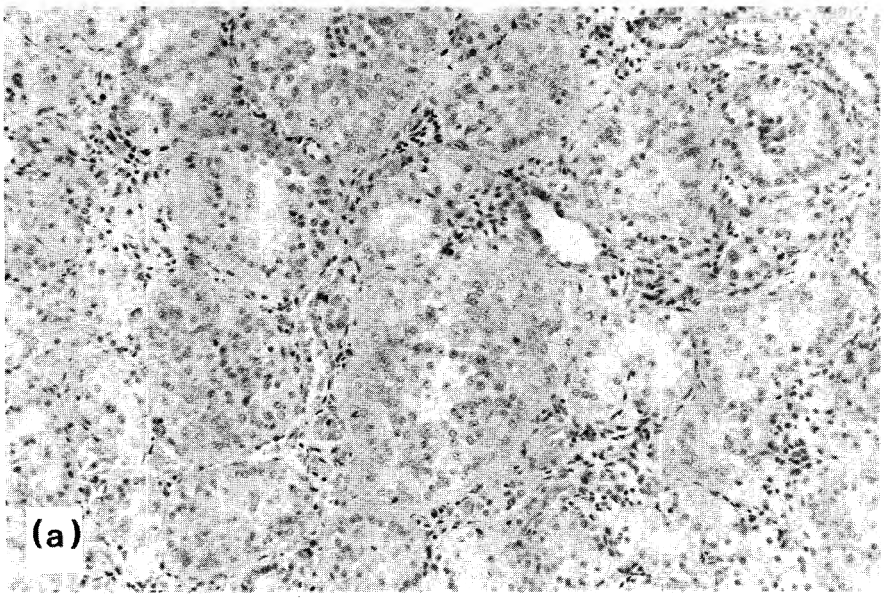

Plate IVa. Renal cortex from a Fischer-344 rat fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr showing a focus of adenomatous hyperplasia. The predominant proliferative lesion is vacuolated. × 75.

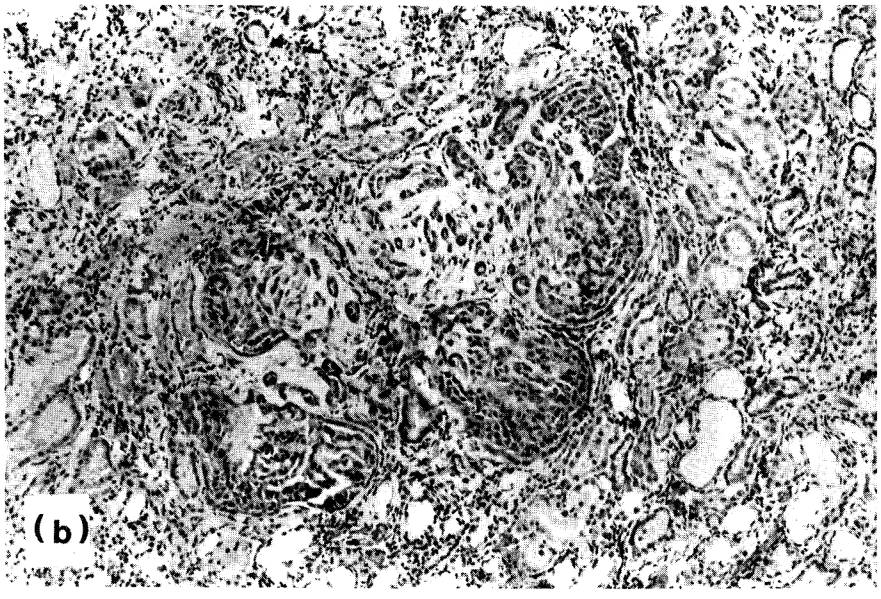

Plate IVb. Renal cortex from a Fischer-344 rat fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr showing tubular cell carcinoma. × 70.

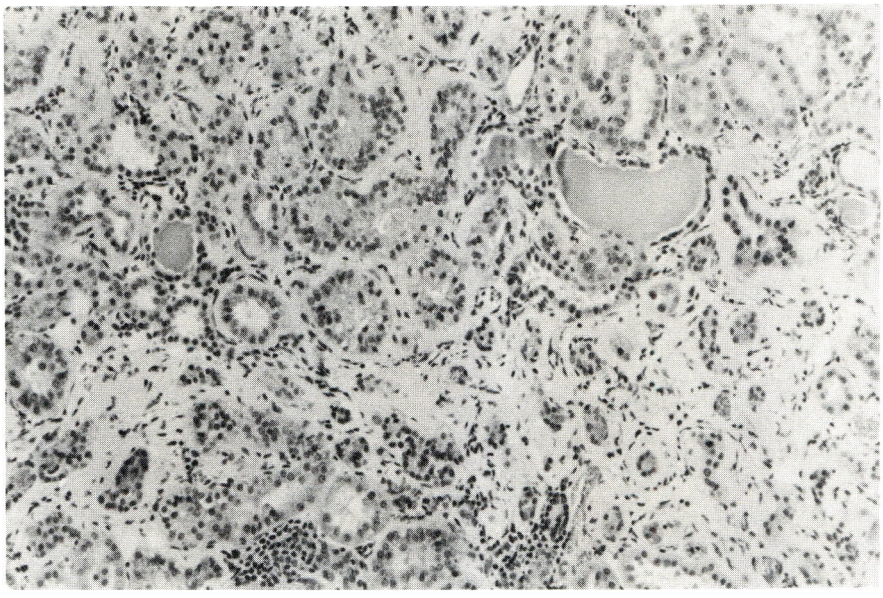

Plate V. Renal cortex of a Fischer-344 rat fed 2% $Na_3NTA.H_2O$ for 2 yr showing moderately severe age-related nephrosis-type lesions diffusely affecting the renal cortical parenchyma associated with tubular hyperplasia of a nonvacuolated cell type. × 75.

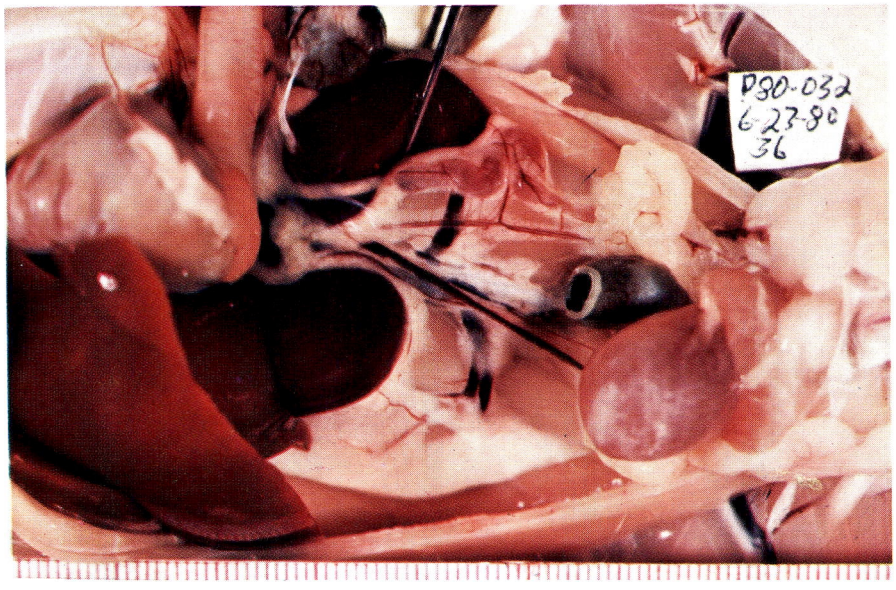

Plate VI. An adult male Charles River (Sprague-Dawley-derived) rat fed $Na_3NTA.H_2O$ at 2% in the diet for 42 days. Note intralumenal blood-tinged urine visible through the serosal surface of the bladder and ureter. The left ureter is dilated. The scale is in mm.

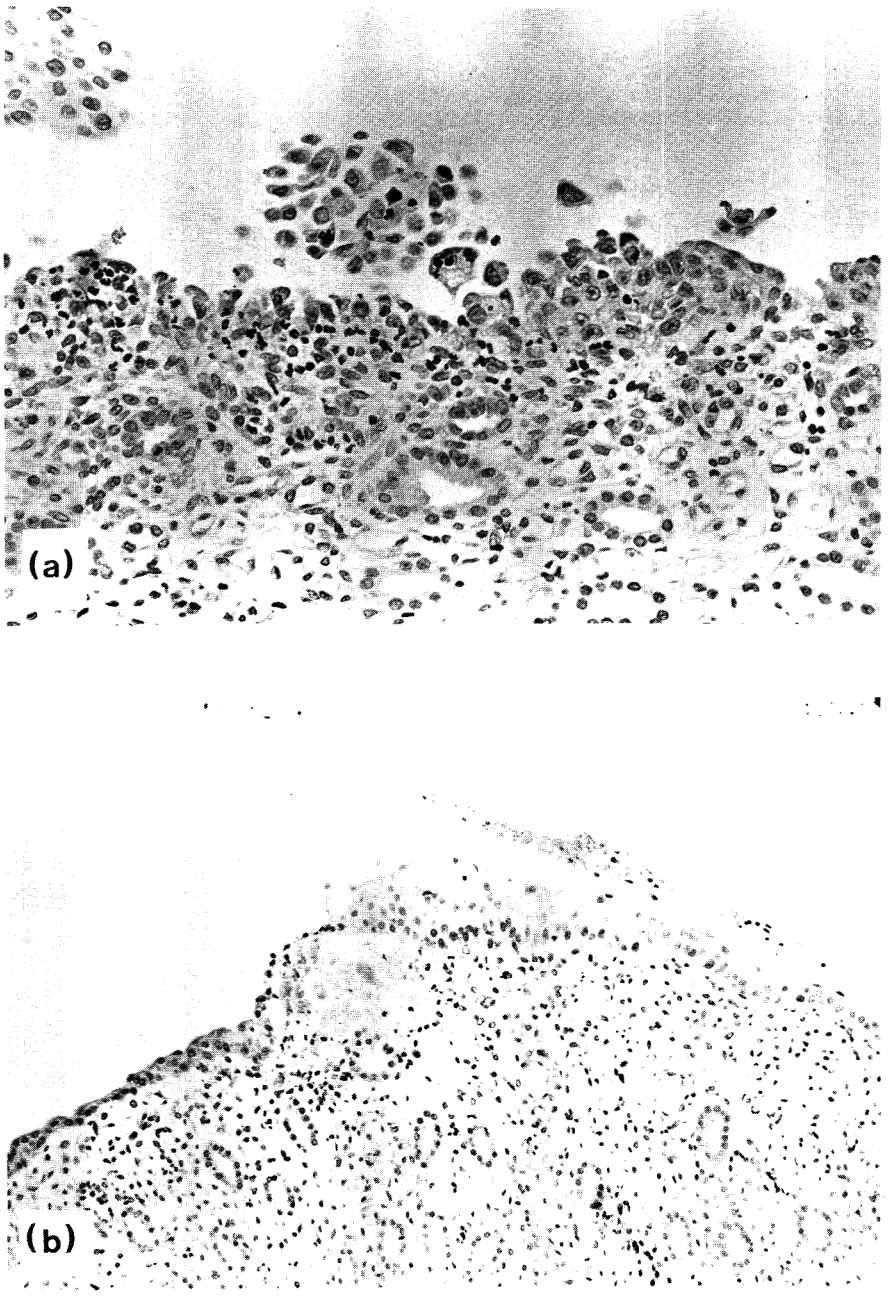

Plate VII. (a) Transitional epithelial erosion in the renal pelvis of a weanling male Charles River (Sprague–Dawley-derived) rat fed $Na_3NTA.H_2O$ at 2% in the diet for 28 days. (b) Ulcerated renal papilla with hyperplasia of epithelium at the periphery of the ulcer. From a weanling male Charles River (Sprague–Dawley-derived) rat fed $Na_3NTA.H_2O$ at 2% in the diet for 28 days. × 145.

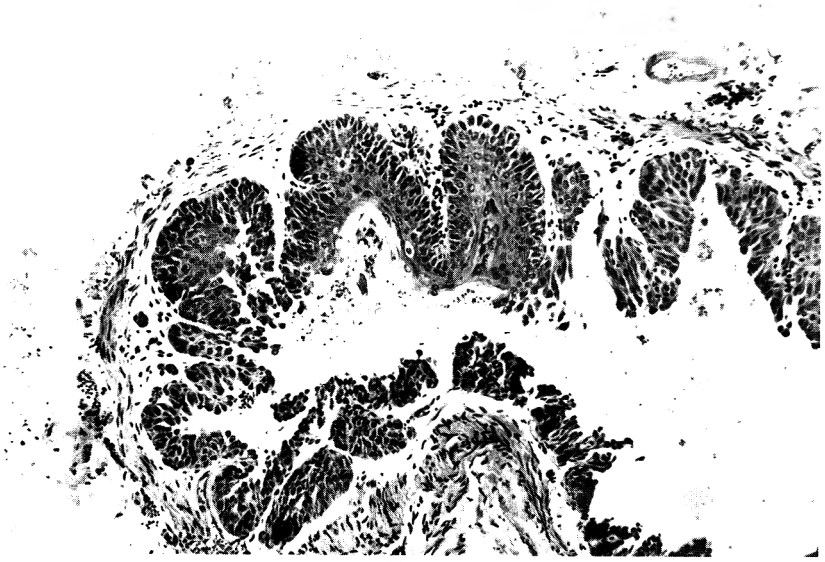

Plate VIII. Endophytic hyperplasia with cellular atypia of ureteral transitional epithelium near the renal pelvis. From a male Fischer-344 rat fed $Na_3NTA.H_2O$ at 2% in the diet for 24 months. × 75.

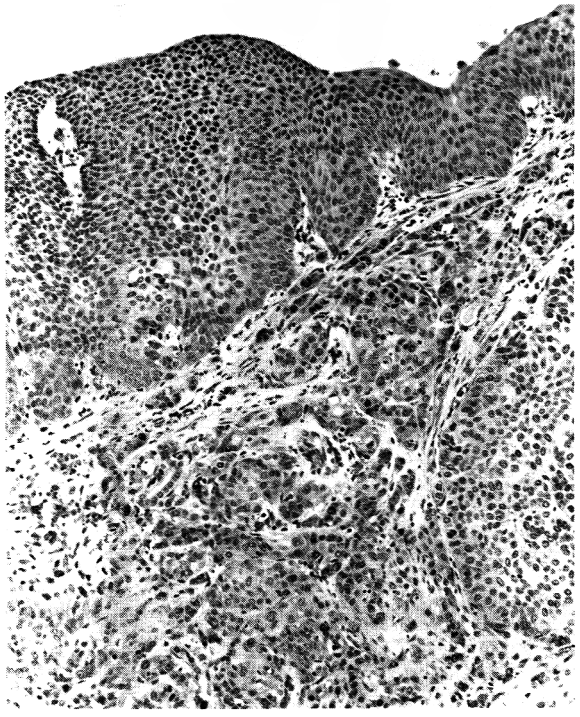

Plate IXa. Invasive transitional cell carcinoma arising in the renal pelvis of a Fischer-344 male rat fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr. × 75.

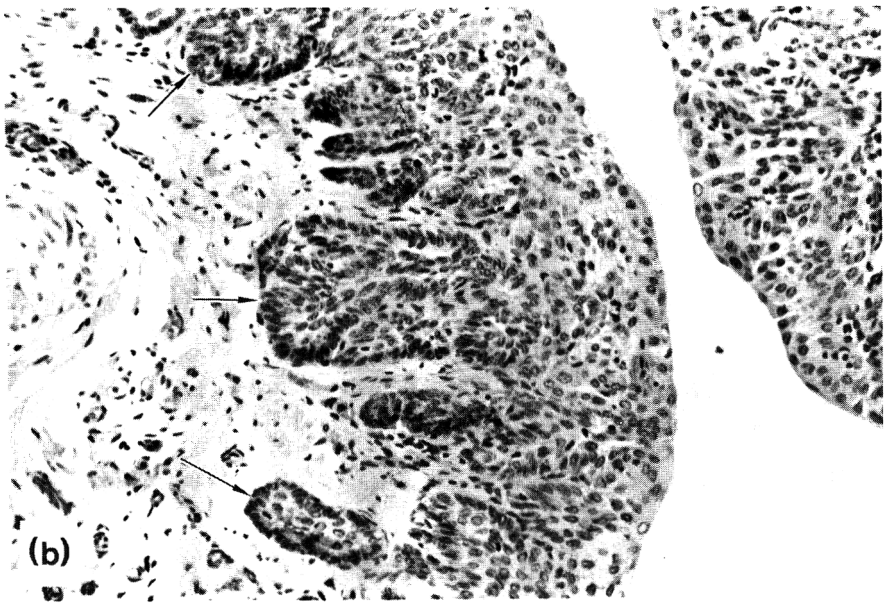

Plate IXb. Endophytic proliferation with cellular atypia (arrows) in the transitional epithelium of the urinary bladder of a Fischer-344 female rat fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr. × 120.

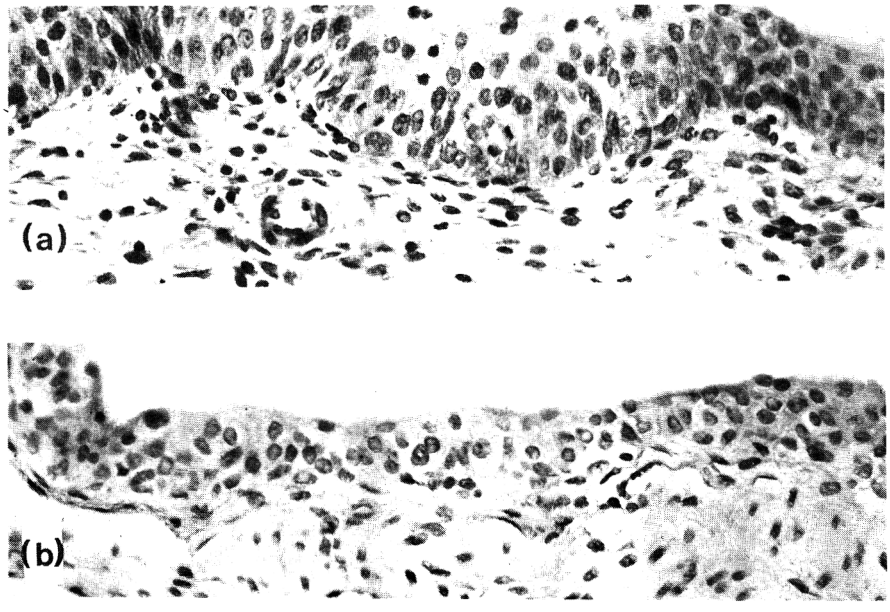

Plate X. Photomicrographs of bladder tissue at sites distant from catheter entry from (a) a rat infused with 400 μmol Na_3NTA/day and (b) a rat infused with 400 μmol $CaNH_4NTA$/day. × 150.

sitional cell tumours are the result of an epigenetic process that involves the disruption of normal cell physiology due to reduced intercellular Ca levels. The reduction in Ca occurs only when the NTA dose is so great (*c.* 40 μmol/g diet) that the urine contains as much NTA as divalent cations on a molar basis. On the basis of this hypothesis it is possible to predict that doses of NTA that do not result in the presence of uncomplexed NTA in the urine will not reduce bladder-tissue Ca levels and will not result in urinary epithelium damage and thus constitute 'no-effect' levels. This hypothesis is clearly consistent with the results obtained in all of the chronic exposure studies reported with NTA (Table 1).

Comparison of NTA dose required to increase urinary bladder carcinoma in rats to human exposure to NTA from drinking-water

The lowest dietary dose of NTA that was associated with urinary tract transitional cell carcinoma in the rat was 0·75% $Na_3NTA.H_2O$ (Table 1) which corresponds to an intake of *c.* 330,000 μg/kg/day in a rat (calculated from data in Table 2). In contrast, humans consuming 35 ml of water/kg/day with an NTA concentration of 2·82 μg/litre ingest 0·1 μg/kg/day or 1/3,300,000 of the lowest dose of NTA associated with bladder carcinoma in chronic exposure studies. The urinary NTA level that must be attained to result in the presence of uncomplexed NTA in the urine ($NTA > M^{2+}$) in man is greater than 140 μmol/kg/day, since man excretes this level of M^{2+} (Altman & Dittmer, 1968). In contrast, the urinary NTA load resulting from the ingestion of water containing 2·82 μg NTA/litre is $0{\cdot}06 \times 10^{-3}$ μmol/kg/day (Table 3), or 1/2,330,000 of the load required to result in uncomplexed NTA in the urine of man.

In summary it is proposed that NTA-associated toxic effects on the urinary tract transitional epithelium are initiated only when urinary NTA attains sufficiently high concentrations so that it extracts Ca from the tissue intercellular spaces. The stripping of intercellular Ca results in a state *in vivo* analogous to a state *in vitro* that induces morphological changes in bladder epithelium (hyperplasia and endophytic growth) similar to that noted after chronic, high dosage NTA ingestion. Finally, evidence is presented that demonstrates that NTA reduces tissue Ca levels only when the NTA concentration in the urine exceeds that of the total M^{2+} in the urine. When urinary $M^{2+} \geqslant$ NTA the tissue Ca level is unaffected and no urinary tract transitional cell damage is induced even after a lifetime of NTA ingestion—that is there is a threshold that must be exceeded. This threshold exceeds human exposure to NTA from the drinking-water by at least six orders of magnitude showing that NTA poses no potential for induction of urinary tract transitional epithelium damage in man.

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PLACENTAL TOXICOLOGY

D. R. GOODMAN, R. C. JAMES and R. D. HARBISON

Department of Pharmacology and Interdisciplinary Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, USA

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Summary—A review is presented of the literature concerning the placenta as a target organ for chemical- and drug-induced injuries that can ultimately lead to teratogenesis or reproductive defects. Such defects can result from the effects of xenobiotics on placental transport, blood flow or pathology, from the metabolism by the placenta of xenobiotics to harmful substances, or from the alteration by xenobiotics of placental endocrine function. Although it is clear that drug- or chemical-induced placental toxicity should be considered as a possible mechanism of teratogenicity, it is an area of research that has been comparatively neglected and is in need of extensive investigation.

Introduction

The placenta is a multifaceted organ that performs a number of essential and highly differentiated functions which are modified throughout the gestation. These functions range from providing a protective barrier that mediates the maternal–foetal exchange of gases, electrolytes, nutrients, wastes and xenobiotics to performing important endocrine functions such as synthesizing gonadotrophins, oestrogens, progesterone, and other steroids. Many of these functions have been studied extensively and their basic physiological or biochemical aspects have been reported. The purpose of this paper is to review the literature regarding the placenta as a target organ for chemical- and drug-induced injury and to emphasize those effects of chemicals and drugs on placental function that can ultimately lead to teratogenesis or reproductive defects.

Effect of xenobiotics on placental transport

The maintenance of proper placental transport of nutrients is essential for normal foetal development and viability, yet the alteration of this function has been comparatively ignored in the study of reproductive developmental toxicology. However, with the slowly growing body of evidence linking the teratogenicity of certain agents to their perturbations of placental transport it seems likely that this potential mechanism will receive more attention in future investigations of the foetotoxic/teratogenic potential of compounds.

Trypan blue had been known to be teratogenic in rats since 1967 (Beck, Lloyd & Griffiths, 1967). However, only recently has it been proposed that the effects of trypan blue on embryos may be mediated by its inhibition of the pinocytosis of large proteins by the rat yolk sac (Williams, Roberts, Kidston, *et al.* 1976). Cadmium is another rat teratogen (Chernoff, 1973) that appears to work by damaging the placenta and disrupting nutrient transmission. Cadmium is much more foetotoxic when it is administered to the maternal organism rather than by direct injection into the foetus even though injection of the maternal organism produces a cadmium burden that is six times higher in the placenta than in the foetus. Furthermore, the level of cadmium reached in the foetus is 20 times greater after direct injection than after maternal injection (Levin & Miller, 1978). Thus the foetotoxicity of cadmium cannot be explained by a direct effect on the foetus but must be mediated by an effect on the placenta. This hypothesis is supported by other investigations in which the effects of cadmium on the transport of zinc and amino acids have been studied. A teratogenic dose of cadmium in pregnant rats results in a 75% reduction in the placental transport of zinc. This loss of zinc lowers the zinc-dependent thymidine kinase activity in embryonic tissue and results in a significantly reduced content of DNA (Samarawickrama & Webb, 1979). That the inhibition of zinc transport and the resulting effects on the foetus, as well as the loss of other important nutrients, may be responsible for the teratogenicity of cadmium is indicated by zinc's antagonism of this teratogenic response (Ferm & Carpenter, 1968).

Alteration of the transport of amino acids across the placenta is another deleterious effect on the developing foetus that is caused by metals. AIB, a non-metabolizable amino acid used in the study of neutral amino-acid transport, has been used extensively in the study of placental amino-acid transport (Fant & Harbison, 1981; Longo, Yuen & Gusseck, 1972; Miller & Berndt, 1974; Rowell & Sastry, 1978; Smith, Adcock, Teasdale *et al.* 1973; Smith, Nelson, King *et al.* 1977). Cadmium, methylmercury and inorganic mercury in micromolar concentrations altered the uptake of AIB in the human placental syncytiotrophoblast microvilli *in vitro* (Goodman, Fant & Harbison, 1978; Goodman, Fant & Harbison, 1979). Cadmium and methylmercury inhibited the rate of AIB uptake while mercury disrupted microvilli integrity. The exact mechanism for the inhibition of AIB transport caused by cadmium and methylmercury remains to be elucidated but recent evidence suggests that there are several important placental processes that the

Abbreviations: AIB = α-Aminoisobutyric acid.

metals may disrupt and these may explain the metal-induced teratogenicity and foetotoxicity.

The uptake of AIB is dependent on both aerobic and anaerobic processes, and inhibitors of oxidative processes such as dinitrophenol inhibit AIB placental transport (Miller & Berndt, 1974) while inhibitors of anaerobic metabolism such as sodium arsenate, sodium fluoride, iodoacetic acid and iodoacetamide also decrease placental transport (Longo *et al.* 1972; Miller & Berndt, 1974; Sybulski & Tremblay, 1967). Strong evidence has also been presented that a Mg^{2+}-dependent, $Na^+ - K^+$-activated ATPase is responsible for the active transport of L-neutral amino acids (Miller & Berndt, 1973 & 1974). This evidence is supported by an inhibition of AIB and glycine transport in the human placenta by ouabain. Besides the energy-dependent regulation of amino-acid transport we have proposed that an acetylcholine-dependent system is involved in transport because of the inhibition of transport caused by high concentrations of nicotine and atropine (Fant & Harbison, 1981; Harbison & Sastry, 1975). Thus, it appears that the metals, which are known to inhibit many enzymatic processes, could affect any one of several enzymatic systems and effect inhibition of amino-acid transport and thereby cause teratogenesis. Likewise, drugs that affect certain other processes involved in the regulation of placental transport can also perturb normal placental transport of nutrients and thereby produce alterations in foetal growth and development.

Effect of drugs and chemicals on placental blood flow

It is difficult to separate effects on placental circulation from effects on transplacental transport processes since the transport processes are in part dependent on blood flow. If the perturbation in blood flow is profound enough, foetal death will result. An excellent example of an agent that causes transplacental transport irregularities and inhibition of placental blood flow is serotonin (5-hydroxytryptamine). Serotonin has been shown to cause a significant decrease in placental blood flow in mice by constricting both arteries and veins (Robson & Sullivan, 1966). There was a dramatic decrease in transplacental transport of radioactive sodium and iodine caused by serotonin which was due at least partly to this effect (Honey, Robson & Sullivan, 1967; Robson & Sullivan, 1966). A single injection of serotonin in mice caused rapid death among the foetuses with little effect on the mother (Poulson, Robson & Sullivan, 1963). Investigators have concluded that foetal death or congenital malformation from serotonin is due to a decrease in placental blood flow and transfer function (Honey *et al.* 1967; Robson & Sullivan, 1966).

Because the effects on placental blood flow can cause deleterious effects on the foetus, drugs used in human pregnancy have come under increasing scrutiny for effects on placental blood flow. β-Adrenergic agonists are often employed to arrest premature labour. Among β-adrenergic agonists, salbutamol and ritodrine caused at least a transitory decrease in human placental blood perfusion (Elnas, Joelsson, Lewander *et al.* 1977; Suonio, Ryynanen, Oklkonow & Castreu, 1976). Ritodrine, however, caused no significant change in blood perfusion of placental tissues of the guinea-pig (Martensson, Sjöquist, Bjellin & Carter, 1979). Negative results were also obtained in the placenta of the guinea-pig with the β-adrenergic agonist terbutaline (Martensson, Sjöquist, Bjellin & Carter, 1978). Sodium triglycyl-[8-lysine] vasopressin, a vasopressin analogue, has been tested clinically in the treatment of upper gastro-intestinal tract haemorrhage. It caused a decrease in placental blood flow in the pregnant guinea-pig and has been suggested, because of this, to be contraindicated in pregnancy (Sjöquist, Bjellin & Carter, 1977).

Cigarette smoking can also adversely effect placental blood flow. Upon smoking a standard cigarette an acute decrease in intervillous blood flow of approximately 15–20% was observed in 12 healthy women. However, the blood flow was back to normal within 15 min after smoking the cigarette (Lehtovirta & Forss, 1978).

Drugs that inhibit prostaglandin synthesis, such as indomethacin, have been shown to have the ability to disrupt normal placental circulation (Rankin, Bersenbrugge, Anderson & Phernetton, 1979). Prostaglandins have been proposed to have an essential role in the regulation of placental circulation in near-term pregnancy (Rankin, 1976). It has been demonstrated in sheep that prostaglandin E_2 can cross the placenta and cause a vasodilating action on the uterine placental circulation and a vasoconstricting action on the umbilical placental circulation (Rankin, 1976). However, upon the administration of indomethacin, a vasoconstrictive action was observed in the umbilical vascular bed, in contrast to the expected vasodilatory actions that would be expected with depletion of prostaglandins (Rankin *et al.* 1979).

Drug- or chemical-induced constriction of blood vessels within the placenta as well as constriction of the umbilical artery or vein has been a subject of investigation since the discovery that placental vessel vasoconstriction can result in decreased blood flow through the placenta and cause foetal hypoxia (Gant & Dyer, 1971). Serotonin, ergotamine and ergonovine constrict sheep umbilical vessels (Dyer, 1969). Dihydroergotamine constricts perfused human placental vessels (Eliasson & Åström, 1955). In perfusion studies using human full-term placenta, morphine, meperidine and codeine have vasoconstrictive action on placental vessels (Gautieri & Ciuchta, 1962). Histamine, digoxin and posterior pituitary extract cause a constriction in the human umbilical artery, while caffeine, aminophylline and sodium nitrite cause a slight dilation (Gokhale, Gulati, Kelkar & Kelkar, 1966).

Since some drugs and chemicals may have effects on placental blood flow, in the future there will be increased emphasis placed on screening drugs for such effects and investigating perturbations of placental blood flow as a mechanism of chemical-induced teratogenesis and foetotoxicity.

Placental xenobiotic metabolism

The 'placental barrier' is fairly limited in the protection it affords to the foetus against the many ex-

posures to xenobiotics that it faces during gestation. Since the placenta provides little hindrance to the transport of most xenobiotics, it becomes very important to understand its function as a detoxifying organ resulting in the enhanced clearance of xenobiotics. There are four possible fates for each chemical absorbed by the placenta: (1) it may be eventually excreted unchanged, (2) it may interact with and compromise the function of the placenta, (3) it may be passed on to the foetus or (4) it may be metabolized. While the second and third events are most certainly potentially foetotoxic, the fourth condition can also have undesirable consequences. However, since the early description of biotransformation processes for increasing the water solubility and hence the excretion of xenobiotics it has become clear that biotransformation may in some instances be beneficial but in others harmful. For example, benzo[*a*]pyrene, a contaminant of cigarette smoke, may be metabolized either to a more water soluble and safer phenolic compound or to a more toxic epoxide intermediate. In the last decade much evidence has been gathered that indicates that the basis of many chemically induced toxicities is the generation of reactive or toxic metabolites. These reactive metabolites have subsequently become the proposed mechanism by which cellular necrosis, mutagenicity, teratogenicity or carcinogenicity might occur. Thus, it seems prudent to understand better the biotransformation capacity of the placenta and determine whether or not it enhances the clearance or the toxicity of xenobiotics absorbed from the bloodstream.

Most of the biotransformation reactions present in the liver have also been found in the placenta (Juchau, 1980). For example, cytochrome *P*-450-dependent reactions, glucuronidation, sulphation, glutathione conjugation, epoxide hydrase, catechol-*O*-methyltransferase and monoamine oxidase all occur or are present in the placenta. However, as is true for most extrahepatic tissues, the biotransformation capacity of the placenta is for the most part many times less than that of the liver. Unfortunately direct comparisons between the liver and placenta for the above-mentioned reactions cannot be made for human tissues nor for any one animal species. However, comparisons of various studies in different species indicate that the placenta appears to have only 3·3 to 2% or less of the activity that is present in liver tissue (Aitio, 1974; Asaoka, Ito & Takahashi, 1977; Juchau & Namkung, 1974; Namkung, Zachariah & Jachau, 1977). The exception may be glucuronyltransferase activity for which the placental activity (Lucier, Sonawane, McDaniel & Hook, 1975) for one strain of rat is equivalent to the liver activity in another strain of rat (Litterst, Mimnaugh, Reagan & Gram, 1975). While this suggests that for the most part xenobiotics are passed comparatively unchanged to the foetus there is increasing evidence that the placenta is capable of metabolizing chemicals with potentially serious consequences.

An interesting example is benzo[*a*]pyrene, an environmental contaminant and a compound found in cigarette smoke and carcinogenic in animals, which is perhaps the compound that has been most extensively examined in placental biotransformation studies. It has been found that benzo[*a*]pyrene metabolism as measured by the formation of 3-hydroxybenzo[*a*]pyrene (a detoxifying pathway) is present at only a low level of activity in nonsmokers but that it may be induced one-hundred fold in smokers (Juchau, 1971). A disturbing feature of this induction of ring hydroxylation, which is also found in humans, is that epoxide formation measured by diol products is also increased while the activity of epoxide hydrase is not (Namkung & Juchau, 1980; Vaught, Gurtoo, Parker *et al.*, 1979; Wang, Rasmussen, Creasey & Crocker, 1977). Further enhancing the disparity between epoxide and diol formation is the finding that the epoxide:phenol ratio is much higher at low substrate concentrations and this suggests that epoxides may be formed in significant amounts *in vivo* (Namkung & Juchau, 1980). An increase in epoxide intermediate in turn would increase the formation of such metabolites as the 7,8-diol-9,10 oxide of benzo[*a*]pyrene which is highly reactive and carcinogenic (Buening, Wislocki, Levin *et al.*, 1978; Kapitulnik, Wislocki, Levin *et al.*, 1978) and thus favour teratogenic events.

In fact, human placental microsomes have been shown to be capable of generating mutagenic metabolites of several polyaromatic hydrocarbons in the Ames assay system (Jones, Fantel, Kocan & Juchau, 1977; Juchau, Namkung, Jones & DiGiovanni, 1978). Thus, it can be concluded that the placenta, like the liver, may be capable of generating reactive metabolites of a number of compounds despite the lower activity of most metabolizing systems and this provides an explanation for teratogenic and embryotoxic effects without the necessity of proposing that the reactive metabolites from the maternal liver must be stable enough to traverse the liver, blood and placental membrane before reaching the foetus and reacting with important macromolecules.

Just how broad a spectrum of chemical activation occurs in the placenta remains to be elucidated. Current evidence suggests that the cytochrome *P*-450 induced by 3-methylcholanthrene is prevalent (Bogdan & Juchau, 1970; Juchau, 1980) but phenobarbital induction has also been observed (Kyegombe, Franklin & Turner, 1973). An induction of placental glucuronyltransferase by TCDD (Lucier *et al.* 1975) suggests that induction by those compounds affecting liver enzymes also affects the inducible placental conjugative pathways. Thus, it appears that those factors affecting liver metabolism and hepatotoxic agents such as multiple metabolic pathways and the induction of toxic *v.* non-toxic pathways, may be applied to explain chemically induced foetal toxicities. Therefore, in the future, placental–foetal biotransformation must be more seriously considered as a biochemical mechanism for chemical and drug-induced foetotoxicities.

Besides the bioactivation of xenobiotics to toxic intermediates it appears that the placental oxidative biotransformation system is capable of compromising foetal viability through its effects on important endocrine functions of the placenta (see later).

Chemical- or drug-induced changes of the placental biotransformation system may enhance the likelihood of placental toxicity and be the mechanism of selected chemical- or drug-induced embryo or foetopathies.

Placental pathology

There are comparatively few studies in animals or humans describing agents causing gross morphological or histopathological changes in the placenta. The placental toxicity of cadmium was investigated in the classical studies of Parizek (1964 & 1965). Upon sc injection of cadmium chloride, acetate or lactate into pregnant Wistar rats between days 17 and 21 of gestation there followed progressive placental changes and destruction. These changes occurred chiefly in the foetal part of the placenta, which was completely transformed within 24 hr into a blood clot with little remaining necrotic tissue (Parizek, 1964). However, in contrast to Parizek's findings, in a recent study, cadmium injected iv into pregnant Wistar rats between days 17 and 21 of gestation was followed by rapid degeneration of the maternal side of the placenta (Samarawickrama & Webb, 1979). In both studies there was an accompanying haemorrhage into the uterine cavity 6–8 hr after treatment (Parizek, 1964; Samarawickrama & Webb, 1979).

The pathological effect of chlorpromazine on the placenta of the CF rat was recently investigated (Singh & Padmanabhan, 1980). A single ip dose of 25–100 mg chlorpromazine/kg body weight during days 8–14 of gestation caused decreases in placental weights of up to 60% and decreases in foetal weight of up to 32%. Histological examination of placentas from treated animals revealed infarctions in the basal and labyrinthine zone. The placental barrier was thicker in chlorpromazine-treated groups. The labyrinthine zone demonstrated a reduction in the foetal vasculature. The trichorial membrane was broken at places causing mixing of foetal and maternal blood. These pathological changes in the placenta and the consequent inadequacy of the foetal circulation may be partly responsible for the terotogenicity of chlorpromazine (Singh & Padmanabhan, 1980).

The effect of smoking on the human placenta is one of the most thoroughly investigated areas of human placental pathology. Several investigators have demonstrated that the ratio between placental weight and birth weight is greater in mothers who smoke than in mothers who do not (Mulcahy, Murphy & Martin, 1970; Wilson, 1971; Wingerd, Christianson, Lovitt & Schoen, 1976). These particular studies did not demonstrate any significant mean placental weight differences between smoking and non-smoking mothers. However, in a more recent study, the placental weights of mothers who smoked heavily (>20 cigarettes/day) were significantly greater than the placental weights of non-smoking mothers matched for period of gestation (Naeye, 1978). Mothers who smoke demonstrate a significant increase in placental disorders, particularly abruptio placentae and placenta previa (Meyer, Jonas & Tonascia, 1976; Naeye, 1979 & 1980). Smokers have thinner, rounder placentas than non-smokers and the distance from the edge of rupture of the membranes to the placental margin is reduced among smokers (Christianson, 1979). Other placental pathological characteristics that occur more frequently in the placentas of mothers who smoke compared to non-smoking mothers include placental calcification, subchorionic fibrin deposits, decidual necrosis and placental infarcts (Christianson, 1979; Naeye, Harkness & Utts, 1977). Smoking-related increases in the incidence of bleeding during pregnancy and in premature and prolonged rupture of the membranes have been demonstrated (Meyer & Tonascia, 1977). Microscopic examination of the human umbilical artery from children of mothers who smoked had pronounced intimal changes when compared with the umbilical arteries of children whose mothers did not smoke. These observations included swelling, bleeding, contraction, and subsequent opening of the endothelial junctions with the formation of subendothelial oedema (Asmussen & Kjeldsen, 1975).

The mechanism of the observed placental morphological changes correlated with smoking has been postulated. Hypoxia in the placenta caused by carbon monoxide and nicotine has been proposed to be the mechanism for the increased placental pathology in smokers (Asmussen & Kjeldsen, 1975; Christianson, 1979).

Chemical- and drug-induced placental pathology may be an important area in safety evaluation studies. As a single adjunct to normal reproductive studies of a given chemical, placental tissues could be routinely examined for gross and histopathological abnormalities.

Xenobiotic alteration of placental endocrine function

The endocrinology of the human placenta is one of the best studied areas of basic placenta function (Thaw & Lamman, 1975). However, the effect of xenobiotics on placental endocrinological function has been practically ignored.

The placenta contains a cytochrome *P*-450 dependent mixed-function oxidase system. The oxidation of cholesterol to pregnenolone proceeds by this system (Simpson & Boyd, 1967) and is the rate-limiting step in the biosynthesis of progesterones. An inverse correlation between aryl hydrocarbon hydroxylase activity and conversion of cholesterol to pregnenolone has been observed in human placentas at term (Juchau, Lee & Blake, 1972). Since there has been a demonstration that cigarette smoking induces aryl hydrocarbon activity in the human placenta, cigarette smokers may run a risk of alteration of hormonal status. In contrast to the conversion of cholesterol to pregnenolone, cigarette smoking causes an increase in the formation of catechol oestrogens by human placentas at term. The placenta mixed-function-oxidase system may catalyse the hydroxylation of both benzo[*a*]pyrene and 17β-oestradiol (Chao, Nelson, Dvorchik & Juchau, 1979). Induction by polycyclic aromatic hydrocarbons in cigarettes may induce a *P*-450-type that hydroxylates 17β-oestradiol to catechol oestrogen at the expense of a *P*-450 type that converts cholesterol to pregnenolone.

The effects of drugs or chemicals on placental endocrine function could result in adverse consequences and alter foetal growth and development. Little information is available about the effects of drugs or chemicals on placental endocrine function and it is clearly an area in need of extensive investigation.

Conclusion

Placental toxicity has seldom been described or studied as a possible mechanism of drug or chemical-induced teratogenicity. The areas reviewed and the multifaceted functions of the placenta make it a highly susceptible target organ for drug- and chemical-induced adverse effects that may subsequently result in abnormal embryo or foetal growth or development or increased foetal wastage. Drug- or chemical-induced placental toxicity should be considered as a possible mechanism of teratogenicity.

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REVIEWS OF RECENT PUBLICATIONS

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.

Vol. 22. **Some Non-nutritive Sweetening Agents.** pp. 208. Sw.fr. 25.00.
Vol. 23. **Some Metals and Metallic Compounds.** pp. 438. Sw.fr. 50.00.
Vol. 24. **Some Pharmaceutical Drugs.** pp. 337. Sw.fr. 40.00.

International Agency for Research on Cancer, Lyon, 1980 (available in the UK through HMSO).

The IARC programme aimed at evaluating the carcinogenic risk of chemicals to man, which has now entered its second decade, is maintaining a commendable productivity, the 1980 additions to the bookshelves derived from the Working Groups' efforts being three separate collections of monographs covering non-nutritive sweeteners, metals and pharmaceuticals.

Many of the Working Groups in the past invited to IARC headquarters in Lyon have had little scope to impress with their interpretative talents. Confident conclusions on carcinogenic status have invariably been precluded by the paucity of available data. The experts assembled to consider the non-nutritive sweeteners saccharin and cyclamate and their major impurities cyclohexylamine, dicyclohexylamine and *ortho*-toluenesulphonamide (oTS), could not complain on this score, saccharin, by way of example, having been the subject of at least 20 oral carcinogenicity studies. Quantity of course is no substitute for quality, but it does help. The Working Group concluded that there was now sufficient evidence that saccharin alone, given at high doses, produces tumours of the urinary tract in male rats and can promote the action of known carcinogens in the bladder of rats. Whilst there was limited evidence that oTS is an oral carcinogen in rats, it was considered unlikely that the levels normally found in commercial saccharin contribute to the observed carcinogenicity of saccharin. In view of the legislative preference given in many countries to saccharin over cyclamate, it is ironic that the Working Group considered the positive carcinogenic status of cyclamate less certain than that of saccharin. The experimental data on cyclamate were said to provide only limited evidence of carcinogencity in rats and mice; there was no conclusive evidence either that cyclamates alone were carcinogenic when given by the oral route or for the carcinogenicity of cyclohexylamine. As with saccharin, there was evidence that the cyclamates can promote the local action of a known carcinogen in the bladder. In addition to the monographs on the individual sweeteners and their impurities a separate summary was prepared on the epidemiological data on the non-nutritive sweeteners as a group. It was concluded that the data taken as a whole did not thoroughly exclude the possibility of a small increase in risk but provided no clear evidence that artificial sweeteners cause bladder cancer in man.

The group of experts that assembled to evaluate the data on arsenic, beryllium, chromium and lead, and their compounds were returning to areas already reviewed by IARC Working Groups some 7 or 8 years earlier. Little change has taken place in the status of arsenic and chromium from that proposed in 1972, namely that sufficient evidence exists to demonstrate that arsenic compounds are skin and lung carcinogens in man and that respiratory cancer is an established risk for men occupationally exposed during chromate production. The 1972 view that there was no evidence that lead salts cause cancer at any site in man has been modified somewhat and the present Working Group felt that lead acetate and phosphate should be regarded as presenting a carcinogenic risk to man. The conclusions about beryllium seem to have been the cause of some controversy. One member of the Working Group dissociated himself from the general conclusion that there was only limited epidemiological evidence that occupational exposure may lead to an increased lung cancer risk, preferring instead the view that beryllium could be considered a confirmed carcinogen in man. The general conclusion of the Group, nevertheless, was that beryllium should be considered suspect of being carcinogenic to man.

The volume of monographs on pharmaceuticals also included compounds that have been reviewed at previous Working Group Meetings, namely phenacetin, phenoxybenzamine and reserpine. In total, 16 monographs were prepared and approved by the 1980 Working Group. In the light of at best, limited animal and insufficient human data, evaluations of human risk proved impossible on clofibrate, dapsone, rifampicin, dihydroxymethylfuratriazine, sulfafurazole, methoxsalen, sulfamethoxazole, hydralazine, phenelzine, spironolactone, phenoxybenzamine and proflavine. Slightly more positive evaluations were possible on phenazopyridine and nafenopin where in each case the animal data supported the view that the drugs should be regarded as human carcinogens. In the case of reserpine it was considered that "a small increase in risk [of breast cancer] cannot be ruled out" although such a risk appeared unlikely. A similar conservatism is apparent in the case of phenacetin. The Working Group considered that there was limited evidence that analgesic abuse leads to cancer of the renal pelvis in humans, but were unable to distinguish which components of the analgesic mixture were responsible.

These monographs are among the required reading of those with an interest in the specific chemicals considered. In addition, the preamble to each volume briefly summarizing the general principles used in the assessment of the carcinogenic risk of chemicals is worthy of a more general audience. This IARC series

is now well established and valued as a source of objective (although admittedly conservative) opinions on carcinogenic status.

Low Level Lead Exposure. The Clinical Implications of Current Research. Edited by H. L. Needleman. Raven Press, New York, 1980. pp. x + 322. $46.24.

This book is evidence of a rapidly changing concern over the levels of lead exposure. Flagrant lead poisoning was common until the late 1950s but then largely disappeared from prominence with the reduction of lead in paints and adjustments in plumbing. Lead at low levels has now been recognized as a widespread public health problem and there is great concern over the effects, especially in children, of persistent exposure to small doses for prolonged periods of time. Early indications of lead poisoning are easily missed since they are rather insidious, being an array of non-specific symptoms, complicating the diagnosis of low-level toxicity.

The first section of the book deals with studies of low-level lead in human populations, with particular regard to effects in children. The effect on neonatal development of prenatal exposure to low levels of lead is considered together with a neuroepidemiological evaluation of children with chronically increased lead absorption. James Burchfiel and his colleagues present good evidence for combining quantitative electroencephalographic measures to evaluate/detect evidence of low-level lead exposure in man. The DHSS Working Party on Lead and Health (HMSO, London, 1980; pp. 129, £4.50) concluded that studies on the effects of low levels of lead in the environment on the behaviour and educational performance of children were inconclusive and ambiguous because of inherent design faults. The evidence presented in this volume for psychological and/or behavioural problems in children is therefore the subject of some controversy. None the less the many recommendations that were made by the Working Party for the identification and reduction or elimination of sources of lead indicate an awareness of the problem. The final chapter in the first section of this book is relevant to this and discusses the management of low-level lead exposure in children.

Several comprehensive chapters comprise the second section of the book and deal with current research. In experiments low doses of lead can be given, under controlled conditions, to experimental animals for prolonged periods of time thus eliminating the variation in exposure between individuals encountered in epidemiological studies on humans. Various effects of lead are reviewed. Amongst these are neurochemical alterations, behavioural changes, alterations in brain anatomy and, perhaps most importantly, the interaction between lead and nutrition.

The concluding section covers another important aspect of lead in the environment by dealing with the legislation, history and current practice of controlling lead pollution. The case for reducing ambient-air concentrations of lead is as keenly argued in these chapters as it has been in the federal courts. The final chapter presents a monetary basis for preventative action by calculating, albeit relatively crudely, the social and economic costs of lead exposure. The high cost ($0.4–1.0 billion annually) of correcting lead-induced health deficits is highlighted.

The format, style and content of this book should appeal to scientists, clinicians and public health officials. It is a valuable contribution to a controversial field and an excellent starting point for those embarking upon investigations of this problem which some consider to be virtually endemic in many urban areas.

Environmental Health Criteria. 13. Carbon Monoxide. Published under the joint sponsorship of the United Nations Environmental Programme and the World Health Organization. WHO, Geneva, 1979. pp. 125. Sw.fr. 10.00 (available in the UK through HMSO).

This document is the product of the WHO Task Group on Environmental Health Criteria for Carbon Monoxide. It is based primarily on original publications listed in the reference section but some recent publications broadly reviewing the health aspects of carbon monoxide are included.

The earlier chapters of the monograph cover the chemistry and methods of analysis for carbon monoxide, its sources, both natural and man-made, its environmental distribution and transformation, environmental levels and exposures including carboxyhaemoglobin levels in the general population, and its metabolism. The following two chapters give a comprehensive review of the effects of exposure to carbon monoxide on experimental animals and on man including sections relating to behavioural changes and work performance. Of special interest are sections detailing high-risk groups including those with cardiovascular and chronic obstructive lung disease or anaemia, individuals living at high altitude, and the embryo, foetus, neonate and infant.

The last chapter discusses the evaluation of health risks and includes the assessment of human exposure from voluntary and involuntary sources. Effects on the cardiovascular and nervous system are considered and the subsequent effects on work capacity.

Recommended exposure levels suggest a limit of carboxyhaemoglobin concentration in the blood of 2·5–3·0% for protection of the general public and those who have impaired health. However, the relationship between concentrations in air and carboxyhaemoglobin levels is affected by several variables, including exposure time and therefore analysis of carbon monoxide in air and the measurement of blood carboxyhaemoglobin levels should be complementary. Air measurements are useful to assist in the planning and implementation of control measures but have limited value in estimating individual carboxyhaemoglobin levels.

As with earlier volumes in this series, the document provides a useful and succinct review of the referenced data. However the recommendations for further studies include surveys of blood levels to assess the magnitude of the problems posed by carbon monoxide in the air of towns, houses and workplaces. Perhaps this should include other possible hazard areas i.e. public meeting places such as cinemas and inns.

The data concerning the effects of exposure to low levels of carbon monoxide for prolonged periods are limited and it is recommended that studies designed to evaluate the possible effects of such exposures and to determine the role of adaptation are needed. The review also emphasizes the need for further studies on the effects of exposure on vigilance and performance in general and in such situations as driving or piloting.

Anionic Surfactants. Biochemistry, Toxicology, Dermatology. Edited by C. Gloxhuber. Marcel Dekker, Inc., New York, 1980. pp. xii + 456. Sw.fr. 130.00.

Because of their widespread use, both by industry and in the home, virtually every individual, at least in the Western world, is continually exposed (directly or indirectly) to low levels of surfactants. For this reason the book cited above, on the biochemistry, toxicology and dermatology of anionic surfactants in warm-blooded animals and man, was a necessary and complementary addition to the ten books that comprehensively cover surfactant chemistry and technology, already published in this series.

Anionic surfactants are, usually for economic reasons, the most frequently used surfactant class. Their activity is dependent on their basic chemical structure, which is characterized by both lipophilic and negatively charged hydrophilic moieties. They have been the subject of ever increasing numbers of toxicological studies in recent years.

Starting with a chapter on the biochemical processes on which the toxicological activity of this class of compounds is based, the details of potential interactions between anionic surfactants and proteins, enzymes and membranes are discussed. The available data on the absorption, metabolism and excretion of these surfactants are then considered in a chapter subdivided by route of exposure, particular attention being paid to studies on percutaneous absorption (*in vivo* and *in vitro*). The results of acute, subacute and chronic toxicity studies in animals, available up to 1977, are then summarized along with observations on the tolerance of anionic surfactants in cases of human ingestion.

A very long chapter devoted to the extensive studies on local toxic effects in animals (which are catalogued in detail) is followed by one on dermatological observations in humans. Next comes a discussion of the carcinogenic, mutagenic and teratogenic activities of these agents, a brief chapter on daily human intakes and a final section dealing with their pharmacological properties.

The chapter entitled 'Daily Human Intake' puts the preceding masses of information firmly into perspective, since the estimated maximum total daily intake for man, by all routes, is 1·0–10 mg, a far smaller amount than the lowest levels at which toxicological effects have been seen in experimental animals, even over extended periods of treatment.

This book provides a comprehensive review of the relevant literature and should prove useful to all those interested in the toxicology of anionic surfactionts.

Chemicals in the Environment. Distribution. Transport. Fate. Analysis. By W. Brock Neely. Marcel Dekker, Inc., New York, 1980. pp. vii + 245. Sw. fr. 82.00.

This interdisciplinary approach to the problems associated with the entry of chemicals into the biosphere is strongly biased in favour of the use of compartmental mathematical models. The use of such models is introduced and their application to various aspects of the problem is described. Coverage is given to determining the movement of chemicals across environmental interfaces, ecological magnification, the application of compartmental models to the environmental distribution of chemicals and the use of mathematical models as an aid in decision making. Despite a gentle introduction the book soon lapses into a terminology that is alien to those with no mathematical leanings.

The concluding chapter considers the problem of informed planning, defining hazard as a function of expected environmental concentration and toxicity. The book is mainly concerned with the prediction of exposure concentrations and hardly touches upon toxicology. Only by the interpretation of environmental chemical concentrations in terms of toxicity can predictions of environmental impact be made. Thus this text provides an introduction to one approach to the problem of persistent chemicals in the environment but will be most useful when complemented by a knowledge of toxicology.

The Principles and Methods in Modern Technology. Edited by C. L. Galli, S. D. Murphy & R. Paoletti. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. viii + 399. Dfl. 122.00.

This volume is the sixth in a series of the results of symposia of the Giovanni Lorenzini Foundation and is the outcome of an international course held in Belgirate, Italy in October 1979. It has an impressive list of contributors but it is difficult to see for whom the volume is intended. Many of the chapters are only repetition of the cook-book formula for conducting safety evaluation studies. This information is widely available and contributes little to the understanding of toxic effects. The experienced reader will find nothing new while the initiate or the general reader will not find justification for the methods proposed.

There are some glimmers of light in the volume. For example the paper by I. C. Munro on reproductive toxicology argues lucidly the need for data on the pharmacokinetic properties of the compound under investigation in order to interpret findings in this complex area of research. He also considers the desirability of *in utero* exposure of animals to be used in carcinogenicity studies and points out some of the problems associated with these experiments such as dose selection and the selection of offspring for the long-term study. The paper by A. K. Palmer on teratology also stands out. Although it says little new about the conduct of the experiment, it shows the value of extensive experience in interpreting the data.

Despite these isolated examples of attempts to approach the problems met with every day the volume in general falls short of the promise of its title.

Experimental and Clinical Neurotoxicology. Edited by P. S. Spencer & H. H. Schaumburg. Williams & Wilkins, Baltimore, 1980. pp. xviii + 929. $132.00.

The publication of this book brings to the fore a field of research that combines synergistically the multidisciplinary approach of toxicology with the work of the clinician, thereby facilitating the extrapolation of experimental data to man. The work is divided into five major sections: "Targets and Classification of Neurotoxic Substances", "Pathophysiological Aspects of Toxic-Metabolic Disease", "Specific Environmental Neurotoxins", "Applied Neurotoxicology", and "Public Issues and Neurotoxicology". Within each of these sections renowned (mainly American) researchers contribute a series of factual and comprehensive review articles.

P. S. Spencer and H. H. Shaumburg propose a concise morphological approach to the classification of neurotoxic diseases which should provide a sound basis for discussion on the development of a "nosology" for neurotoxic disease. J. M. Jacobs discusses in depth the relationship between vascular permeability and neural injury with a factual account of its role in both clinical and experimental situations. This is an important contribution because reversible disruption of the blood–brain barrier has recently been recognized not only as a toxic effect but also as a technique that may have clinical use in the delivery of lipid insoluble compounds to the brain. Evidence for this is provided by the recent findings that the delivery of enzymes or cytoreductive chemotherapeutic agents may be greatly improved using hypertonic mannitol to modify cerebral capillary permeability (Neuwelt *et al.* 1981, *Neurology* **31**, 45 & 86) and such techniques may open up new possibilities in the therapy of CNS disorders.

M. I. Sabri and P. S. Spencer describe biochemical models and hypothetical mechanisms for toxic distal axonopathy with particular reference to organophosphorus compounds such as tri-*o*-cresyl phosphate on the one hand and carbon disulphide, acrylamide and 2,5-hexanedione on the other.

There is a series of excellent chapters documenting the neurotoxicity of selected metals, organophosphorus compounds, drugs and various chemicals and biological toxins. The chapter on lead is of special significance since legislative measures to restrict its use currently outstrip the science. M. R. Krigman, T. W. Bouldin and P. Mushak approach the plethora of literature on this subject meticulously but still find it difficult to translate the basic toxic effects of lead into the observed syndrome. The time seems ripe for collaboration between clinicians and laboratories to produce a controlled approach to this ubiquitous problem.

The methyl *n*-butyl ketone story is discussed first by P. S. Spencer, D. Couri and H. Schaumburg and later by N. Allen who describes its identification as a neurotoxin. Other articles cover the *in vivo* experimental neurotoxicity of its metabolites and most interestingly the use of an organotypic tissue culture system to reproduce and analyse methyl *n*-butyl ketone neuropathy. The extension of this approach must be of great value to future research into neurotoxicological mechanisms.

A paper by T. Damstra and S. C. Bondy indicates some possible avenues of investigation by considering the use of biochemical assays to study the disruption of neuronal enzyme systems.

The volume is excellently presented with clear concise figures and tables. The reproduction of the many photographic plates is of a good quality, and extensive reference sections are included for all of the topics covered. The emphasis is without doubt and rightly towards a neuropathological approach. Nevertheless this work should have a place in every toxicological institution and will prove to be of great value to both clinicians and experimental neurologists who require a comprehensive introduction to this exciting field of research, as yet in its infancy.

Carcinogenesis: Fundamental Mechanisms and Environmental Effects. Edited by B. Pullman, P. O. P. Ts'o & H. Gelboin. D. Reidel Publishing Company, Dordrecht, 1980. pp. xi + 592. Dfl. 120.00.

The admirably rapid publication of the proceedings of the Thirteenth Jerusalem Symposium on Quantum Chemistry and Biochemistry held in April/May 1980 provides workers in the field of chemical carcinogenesis with an up-to-date summary of the current thinking of many of the most well known scientists on the subject.

Although much of the thinking may be painfully preditable, the excellent quality of much of the experimental work described immediately recommends close attention. All too often the conclusions reached by the authors seem to be dictated as much by conventional theories as by the results of their elegant research. Nevertheless the overall picture created by the often conflicting conclusions in this collection of papers is far from clear cut. Valiant attempts by the theoretical giants [H. C. Pitot, L. Sachs, P. O. P. Ts'o] to bring some order into the chaos are inevitably hindered by the current limits of our knowledge in this field.

The ultimate test of a book of this kind must be the extent to which it reflects the state of development of the subject that it covers. Thus the success of the book depends heavily on the judgement of the conference organizers. In this case the reflection is accurate although the image is fragmented and confused—a perfect reproduction of an imperfect stage in the maturation of a science.

The general presentation of the book is good for such a rapid publication but it could do with a better subject index. Some attempt to divide the 47 papers into separate sections would have been useful although it might have been a daunting task.

Testicular Development, Structure and Function. Edited by A. Steinberger & E. Steinberger. Raven Press, New York, 1980. pp. xx + 536. $70.72.

This volume represents the proceedings of the Sixth National Institute of Child Health and Human Development Workshop on the Testis which was held in Texas in March 1979. It contains fifty-six manuscripts from world-renowned authors engaged in a variety of

disciplines, together with discussions. The editors have divided the presentations under five main headings covering the more recent advances in: testicular development and differentiation, spermatogenesis and steroidogenesis and their hormonal control, membrane transport and protein secretion, cyclic changes in testicular function and, lastly, sperm motility and energetics.

The first section covers a host of topics varying from a review on the development of the testis and male reproductive trace to studies on the development and function of the blood–testis barrier in rats and mice. The second section contains seventeen papers dealing with spermatogenesis and steroidogenesis both in the interstitial and germ-cell compartments from a variety of species with particular reference both to their direct hormonal control and to their control via the hypophysis.

Section three, concerning membrane transport and protein secretion, deals with androgen binding and transport of steroids in the testis together with presentations on calcium regulation and microtubule–membrane interactions.

Cyclic changes in testicular function occurring in animals as diverse as the rock hyrax, the rhesus monkey and the hamster are dealt with in the fourth section. Particular reference is made to the effects of seasonal change and diurnal rhythms on testicular function. The final section contains ten papers covering the general topic of the control and requirements for sperm motility.

The volume is extremely well presented with a comprehensive subject index. Contributions are of a uniformaly high standard. The editors should be congratulated on the incorporation of these recent studies from such diverse scientific fields in the one volume. This book should be of great interest to those engaged in studies or research concerning the male reproductive system.

Delayed Hypersensitivity. Research Monographs in Immunology. Vol. 1. By J. L. Turk. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. xii + 295. Dfl. 135.00.

This monograph (a third revised edition) portrays Professor Turk's experience of the subject of delayed hypersensitivity over the last 20 years, which he puts into the context of existing knowledge as well as indicating future lines of research. It is characterized throughout by a most admirable lucidity.

The impetus for the study of delayed hypersensitivity came first from those involved in research on tuberculosis and bacterial allergy, then from Landsteiner and his colleagues working on chemical-contact sensitivity and, more recently, from the interest in tissue transplantation and tumour immunology. The most exciting development has been the increasing awareness that research in these three areas is concerned with the same basic biological phenomena, in which there are many events in common, but with a number of different consequences. The reaction between sensitized lymphocytes and antigen may result in the direct cytotoxic action of the lymphocyte, the elimination of infecting organisms by activated macrophages, or the release of pharmacological agents that can cause local tissue damage.

It is the local tissue-damage reaction, which cannot be separated from other cell-mediated immune reactions, that is known as delayed hypersensitivity, and the induction of delayed hypersensitivity and the histology of delayed hypersensitivity in the skin are discussed in the two chapters that follow the informative and concise introduction. For delayed hypersensitivity produced by contact with a chemical sensitizing agent (a subject of particular interest to toxicologists) degenerative changes in the epidermis are found early on, especially in relation to the infiltration of the tissue with mononuclear cells. The sequence of events is the immunological reaction on a tissue surface, tissue damage and then secondary inflammatory features. Professor Turk points out that the cellular infiltrate in delayed hypersensitivity reactions is not specific to the hypersensitivity but is determined by the manner in which a certain tissue of a particular species reacts to an irritant process of the same intensity and over the same period of time. A comparison of the cellular infiltrates in delayed hypersensitivity reactions in the guinea-pig and human is used to emphasize this point.

The passive transfer of sensitivity, which implies that sensitized cells can transfer the ability to mount specific sensitivity reactions in naïve recipients, is discussed in relation to both experimental animals and man. The use of cyclophosphamide to increase contact reactivity and certain other forms of delayed hypersensitivity by elimination of suppressor cells, is discussed in a chapter on immunoregulation along with various models of immunological unresponsiveness to both protein antigens and chemical sensitizing agents.

A most interesting chapter deals with changes in lymphoid tissue during the induction of delayed hypersensitivity, and includes discussion of structural changes occurring in the local draining lymph nodes and cellular events such as the production of new cells and the metabolic activity of the immunoblasts. Another chapter deals with the concept of carrier-protein specificity and from some of the studies described it appears that the greater amount of antibody-like activity in delayed hypersensitivity only reacts with the right hapten on the right carrier, and that both hapten and carrier specificity may exist together.

In the last chapter Professor Turk discusses evidence that is now beginning to accumulate suggesting that delayed hypersensitivity may parallel inflammatory tissue changes and be involved in granuloma formation leading to fibrosis. Delayed hypersensitivity reactions and granuloma formation could frequently be directed to the same antigens in a tissue or infecting organisms and a study of delayed hypersensitivity is thus a model for the understanding of a wide range of chronic inflammatory diseases.

This book emphasizes that studies of delayed hypersensitivity are especially difficult since no chemical intermediary, neither antibody nor pharmacological agent, has yet been completely characterized. All work has to be done using live animals or living cells in tissue culture. In spite of the problems, however, researchers in this field have been at the forefront in defining the cellular regulation of the immune re-

sponse and its modifications by immuno-modulatory drugs.

Thymus, Thymic Hormones and T Lymphocytes. Edited by F. Aiuti & H. Wigzell. Academic Press, London, 1980. pp. ix + 445. £25.00.

This book reports the proceedings of a Serono Symposium that was particularly concerned with research into thymic hormonal factors and their influences on the differentiation and functions of T lymphocytes.

The first section deals with the morphological and biological aspects of the thymus. Experimental studies, particularly in mice, have clarified certain aspects of thymic development. The reticular epithelial cells of the thymus are firmly established as producing humoral factors, and recent findings demonstrate that they, and probably also macrophages, express large amounts of antigens of the major histocompatibility complex. Thymus-derived lymphoid cells may therefore 'learn' to recognize antigens of the major histocompatibility complex within the thymus. An interesting chapter extends these observations to human thymocytes and also demonstrates that similar reticular cells are found in regional lymph nodes. The authors postulate the thymic 'education process' for thymocytes is repeated on the periphery where T lymphocytes are exposed to antigenic stimuli, a hypothesis borne out by work on human T cells presented by other workers at the Symposium.

The next section is devoted to studies on natural killer (NK) cells and T lymphocytes in normal and pathological conditions and one of the problems discussed is the relationship between these two types of cells. NK cells are endowed with cytolytic ability mediated via contact with the target cells. They can mature in the absence of a thymus, but evidence was presented indicating that NK cells are prethymic cells in the T-cell lineage and it has been demonstrated that their activity can be augmented by interferon.

The last two sections concern thymic hormones and the clinical and immunological effects of thymic hormones in immuno-deficiency diseases. At least eight thymic hormones have been isolated since 1966. Several polypeptide hormones have been isolated from thymic tissue, and others have been isolated from blood and thymic epithelial cell cultures, suggesting the existence of several distinct thymic factors. A large number of researchers in this field discuss their findings and the use of the hormones to study thymic function. Defects in the thymus gland apparently lead to the relatively rapid loss of the short-lived T cells responsible for controlling the immune response; other evidence strengthens the case of the differentiation and maturation of T cells being under control of a thymic hormone. *In vitro* studies and limited clinical trials in patients with primary or secondary T-cell defects have indicated that thymic hormones are capable of restoring or partially restoring cell-mediated immune responses. Patients with chronic bacterial infections, serious viral infections and with neoplasms have also been treated with various thymic humoral factors, although the number of such patients is small, and whether these hormones definitely react outside the thymic tissue in adults is not clear.

These proceedings make fascinating reading. The Symposium brought together immunologists working in the same field, but using different techniques and approaches to the problem of why and how T lymphocytes differentiate. It is sobering to realize, in view of all these data, that the thymus was only recognized as an immunological organ in 1961.

Experiments with Normal and Transformed Cells. A Laboratory Manual for Working with Cells in Culture. By R. Crowe, H. Ozer & D. Rifkin. Cold Spring Harbor Laboratory, New York, 1978. pp. 175. $19.80.

Mammalian cells maintained in cell culture can be transformed by both viruses and chemical carcinogens. Once transformed these cells display many of the characteristics of cancer cells. Growth controls shown by normal cells in culture such as anchorage dependence and contact inhibition are lost. There are also biochemical differences between normal and transformed cells such as the high level of protease plasminogen activator produced by transformed cells. Once transformed *in vitro*, most cells will produce tumours when injected into animals.

Because of the characteristics they share with cancer cells, transformed cells provide a useful model for looking at the mechanisms of carcinogenesis and are therefore used in many laboratories around the world. *Experiments with Normal and Transformed Cells* is exactly what it purports to be; it is laid out as a laboratory manual composed of a series of experiments performed during a 'Transformed Cell Course' at Cold Spring Harbor Laboratory in 1978. It is therefore essentially a technical book but it also provides a certain amount of background information to each set of experiments. Although the experimental methods are comprehensively described, some experience with cell cultures would be required before embarking on any of these techniques.

The standard cell-culture methods described include the preparation of primary cultures from both mouse and chick embryos and their subsequent infection by retroviruses, which include both sarcoma and leukaemia viruses. Normal and transformed cell lines are used to demonstrate the different growth control properties obtained *in vitro* by transformed cells and there is a short section on chemical mutagenesis which covers the induction and selection of mammalian cell mutants.

The book then progressses to more sophisticated methods such as the generation and selection of cell hybrids and immunofluorescent staining for viral antigens in virally transformed cells. Infection of cells by DNA, an assay for plasminogen activator, and karyotype analysis are also covered. Towards the end of the book the techniques become more biochemically orientated and include the investigation of cell-surface proteins, immunofluorescent staining of internal proteins, induction of macromolecular synthesis and the uptake of precursors in resting cultures and, finally, the preparation of cytoplasts.

Although *Experiments with Normal and Transformed Cells* is very much geared to the field of

tumour virology there are some useful methods which can be applied to cultured cells used in many aspects of research. The only criticism that can be made is that since the book is written as a manual for a practical course and presumably many of the experiments are designed to run simultaneously, the order of topics covered is not necessarily the most logical.

An Introduction to Spectroscopy for Biochemists. Edited by S. B. Brown. Academic Press, London, 1980. pp. xv + 403. £16.60.

Dr Brown has taken on the formidable but no doubt absorbing task of compiling a single volume that deals with various spectroscopic techniques and is aimed at the non-specialist biochemist. He has also inadvertently produced an excellent means of revision for the chemist who has temporarily lost touch with practical spectroscopy.

The book begins with a short general introduction to spectroscopy and a simple explanation of energy levels and transitions. Seven chapters follow covering ultraviolet and visible spectroscopy, molecular emission spectroscopy, vibrational spectroscopy, circular dichroism and optical rotation, nuclear magnetic resonance, electron paramagnetic resonance, atomic absorption spectroscopy and mass spectrometry. Mass spectrometry, although perhaps not strictly a spectroscopic technique, is included because of its increasing importance in investigative biochemistry. I feel that a short chapter on Mössbauer spectroscopy should have been included for the sake of completeness, although the editor does mention the omission in the preface.

The same general format is followed in each chapter. This includes an introduction, followed by some theoretical principles and instrumental considerations. There are often cross-references to other spectroscopic techniques but the advantages of the particular technique under discussion are outlined and examples of molecules or compounds that lend themselves to investigation by that technique are given. Useful indications of range and sensitivity are included, and the reader is made well aware of the possible difficulties involved in sample preparation and interpretation of results. The contributors have avoided the usual pitfall of 'selling' their particular technique, and are content to list some of the major achievements and to suggest further possible applications. A list of reviews and further reading accompanies each chapter.

The overall result is a very readable 'armchair' reference book, the chapters on ultraviolet and visible spectroscopy and mass spectrometry being particularly clear to the non-specialist. The mathematical content should not prove troublesome, while the diagrams of instrumentation and spectra are amongst the best to be found.

An additional chapter would have been worthwhile. This could have given the reader a flow diagram, decision tree or simply an ordered list of hints on how to approach the investigation of samples of unknown structure and have suggested the techniques that might initially prove most informative, the interpretation of initial results and which secondary techniques to bring into play.

The production of this book is of high quality, and most biochemists and undergraduate science students would find it extremely useful, particularly if, when the need arose, they could call upon the expertise of a specialist spectroscopist.

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Information Section

ABSTRACTS AND COMMENTS

Chromosome damage from caramel

Stich, H. F., Stich, W., Rosin, M. P. & Powrie, W. D. (1981). Clastogenic activity of caramel and caramelized sugars. *Mutation Res.* **91,** 129.

Long-term feeding studies on various types of caramel have revealed no evidence of carcinogenicity, although ammonia-process caramel produced lymphocytopenia at all levels tested (Evans *et al. Fd Cosmet. Toxicol.* 1977, **15,** 523). A food-grade caramel also gave negative results in an Ames test with five Salmonella strains (Bonin & Baker, *Fd Technol. Aust.* 1980, **32,** 608). However, a very weak mutagenicity of caramel to *S. typhimurium* TA100 was reported elsewhere, and several other studies have demonstrated the mutagenicity of sugar pyrolysates (Sugimura *et al. CRC Crit. Rev. Toxicol.* 1979, **6,** 189). A sugar-ammonia model system, the reaction products of which included many pyrazines, was also strongly mutagenic (Spingarn & Garvie, *J. agric. Fd Chem.* 1979, **27,** 1319).

In the present study Chinese hamster ovary cells were exposed for 3 hr to commercial caramel powder (a positively-charged material prepared by heating a sugar–ammonium solution), or to caramelized solutions of sucrose, fructose, glucose, mannose, maltose or arabinose (prepared by heating solutions at 180°C for 1 hr). The caramel and all the caramelized sugars induced a high frequency of chromosome breaks and exchanges, the potency of caramel itself (which produced 46% metaphase plates with chromosome aberrations) being exceeded only by caramelized fructose. Non-caramelized sugars did not increase the aberration frequency. The transition metals Cu^{2+} and Mn^{2+} had no effect on caramel's potency, but Fe^{2+} and Fe^{3+} had a strong inhibitory effect. Addition of an S-9 microsomal preparation from PCB-pretreated rats abolished the clastogenic activity of the caramel powder, and this property was unimpaired by removal of NADP or $MgCl_2$ from the S-9 mixture. However, heating for 20 min at 80°C did destroy its inhibitory action. It was concluded that the effects of the S-9 preparation were not due to the mixed-function-oxidase system, but might result from a physical binding of caramel to S-9 components.

No attempt has yet been made to identify the clastogenic ingredients of caramel, although possible candidates may be furan and its derivatives or maltol. As yet unpublished studies by the authors have shown some of the former to be mutagenic in more than one test system, while the latter induced reverse mutations in *S. typhimurium* strain TA100 (Bjeldanes & Chew, *Mutation Res.* 1979, **67,** 367).

[Whether these *in vitro* findings have any significance to caramel consumption by man is still open to question. If its potency is abolished by binding to S-9 components, it may similarly be abolished by binding to dietary components.]

Yet more coffee-drinking rats

Nolen, G. A. (1981). The effect of brewed and instant coffee on reproduction and teratogenesis in the rat. *Toxic. appl. Pharmac.* **58,** 171.

There have recently been a number of reports on the hazards linked with caffeine and its associated drinks. In the last issue (*Cited in F.C.T.* 1981, **19,** 789) we reported a paper linking coffee with pancreatic cancer and earlier we detailed studies that indicated possible teratogenic effects in the rat (*Food Chemical News* 1980, **22,** (26), 35; *Cited in F.C.T.* 1981, **19,** 510). We have previously commented (*ibid* 1981, **19,** 510) that care should be taken in drawing conclusions from these studies since it is possible that the rat is uniquely unable to cope with caffeine stress. It is, therefore, rather disappointing to find yet another study using the same species and providing little extra information.

Brewed or instant coffee containing at full strength 0·057 and 0·045% caffeine, respectively, was given to groups of 20 male and 30 female rats in place of their drinking-water either at full strength or as 50 or 25% dilutions. Control animals received distilled water. Administration continued for about 30 wk, from weaning through two pregnancies. The initial mean daily caffeine intakes of the females were 181, 98 and 57 mg/kg body weight in the high-, medium- or low-dose brewed coffee groups, respectively, and 148, 81 and 45 mg/kg, respectively in the groups given instant coffee. During lactation the daily caffeine intakes were 97, 53 and 30 mg/kg in the rats given brewed coffee and 80, 46 and 22 mg/kg in those given instant coffee. Ten animals from each group were killed at 13 wk and examined. The remaining rats were mated and the resulting pups were culled to eight/litter. Ten days after their first litter had been weaned the females were mated again and killed at either day 13 or 21 of pregnancy. The pups in the first litter were weighed and then discarded when 21 days old. The foetuses from the second mating were examined for any abnormalities.

Rats given full-strength coffee tended to drink less than those given the diluted brew but all the coffee-drinkers grew as well as or better than the controls. All of the rats given 100% instant or brewed coffee solutions had enlarged kidneys at 13 and 30 wk but at the 50% dilution only the females showed any kidney enlargement. The females were also more susceptible with regard to their livers, full-strength coffee producing an enlargement at both 13 and 30 wk in females but not in males, and 50% coffee solution producing this effect in females at 13 wk. No effects on organ weights were seen in the low-dose group.

No effects of coffee ingestion on reproduction or lactation were observed except for a significant reduction in the body weights at weaning of the pups of dams given 100% coffee solution. Neither were any

embryotoxic or teratogenic effects noted even in the groups given the full-strength coffee which provided caffeine intake equivalent to that (30 mg/kg body weight) at which ectrodactyly (missing toes) occurred in an FDA study (*Food Chemical News* 1980. **22**, (26), 35). However, delayed ossification of the sternebrae was noted, but only in the groups given 100 or 50% brewed coffee solutions or 100% instant coffee solution. In the FDA study this effect occurred even at the lowest dose level of 6 mg caffeine/kg.

[In the FDA study (*Food Chemical News* 1980, **22** (26), 35) the caffeine solutions were administered by gavage. A recent study using [^{14}C]caffeine has indicated differences in blood levels of caffeine and in the relative distribution of ^{14}C in the blood and placenta between rats given caffeine solution by gavage and those sipping the solution (*Food Chemical News* 1981, **23** (9), 32). Differences in dosing methods may therefore explain some of the differences in the results of the two studies mentioned above.]

Living it up on Watership Down

Klurfeld, D. M. & Kritchevsky, D. (1981). Differential effects of alcoholic beverages on experimental atherosclerosis in rabbits. *Expl molec. Path.* **34**, 62.

The debate on the possible health benefits of a moderate intake of alcohol needs little introduction. The arguments have continued for a long time, coloured not a little by the conflict between the view that "a little of what you fancy does you good" and the more puritanical assumption that if it's enjoyable it must be bad—in one sense or another. While there is no doubt about the adverse effects of high intakes of ethanol in any form, claims for the health-promoting effects of moderate drinking make the news from time to time (*Cited in F.C.T.* 1981, **19**, 739), and divergent assertions on the relative merits of various types of alcoholic beverage as a protection against coronary heart disease led the authors cited above to study the relationship between alcohol consumption and atherogenesis in animals.

The study they describe compared the effects of a moderate intake of various alcoholic drinks on the development of atherosclerosis in rabbits fed a semi-purified diet, which was formulated to simulate "the average American diet" and was supplemented with additives to meet the rabbit's nutritional needs and with sufficient cholesterol (0·5%) to induce atherosclerosis rapidly. Groups of eight rabbits were fed this diet for 3 months together with one of the following drinking fluids: 12·5% glucose in water (control group), red wine, white wine, bourbon whiskey, beer or ethanol in water. The beer contained 4·9% ethanol; the other fluids were diluted to 9·5% ethanol. At the end of the treatment, and after an overnight fast, the animals were killed for blood studies and histopathology. Fluid consumption, recorded daily, was significantly below the control intake in all the test groups except that given beer (33–46 ml/day for the test groups *cf.* 73 ml/day for the controls). Weight loss in all the animals, including the controls, was attributed to the relative unpalatability of the diet although the weight loss in the red-wine and white-wine groups was significantly greater than that in the control group. Total serum lipids were similar in all the groups.

None of the ethanolic fluids caused liver enlargement or exacerbated the hepatic-lipid accumulation caused by the diet. Hepatic levels of cholesteryl esters were actually lower in the treated groups than in the controls. Again in comparison with the controls, both aortic and coronary atherosclerosis were considerably reduced by consumption of red wine and showed some reduction in the groups given ethanol–water, white wine or whiskey, but beer was ineffective in this respect. The aortic lesions were of two types: fatty streaks composed almost entirely of foam cells, and a more advanced type of atheroma characterized by proliferation of smooth muscle cells in a loose collagenous matrix. Some animals (about 20%) had both types of lesions, but the former type was more common in the control and red-wine groups and the latter predominated in the other test groups. Analyses of serum and liver for high- and low-density lipoprotein (HDL and LDL), showed that HDL-cholesterol and HDL-phospholipid concentrations were greatly increased by all the alcoholic fluids except beer. Consequently there were significant reductions in LDL/HDL ratios and a marked increase in the percentage of total cholesterol contributed by HDL. Discussing this the authors refer to evidence that levels of HDL cholesterol may be inversely related to morbidity and mortality from coronary heart disease.

While the mechanisms underlying these findings require further investigation, this study provides support for the view that the consumption of alcoholic beverages is not atherogenic and may exert a protective effect. The varying effects of the different types of beverage indicate the involvement of constituents other than ethanol in the overall response.

The effects of dietary cyanide

Tewe, O. O. & Maner, J. H. (1981). Long-term and carry-over effect of dietary inorganic cyanide (KCN) in the life cycle performance and metabolism of rats. *Toxic. appl. Pharmac.* **58**, 1.

Chronic cassava consumption by malnourished human populations is associated with a distinctive clinical syndrome, which includes degenerative neurological disease and endemic goitre (*Cited in F.C.T.* 1980. **18**, 445). These effects are caused by cyanogenic glycosides such as linamarin, present in the cassava and hydrolysed *in vivo* to cyanide, coupled with chronic nutritional deficiency. The latter factor may involve shortages of sulphur-containing amino acids, which normally assist the enzyme rhodanase in detoxifying cyanide, and/or vitamin B_{12} (*ibid* 1970, **8**, 606 & 711; *ibid* 1975, **13**, 157). No thyroid lesions were observed in rats fed nutritionally-balanced fresh cassava diets containing 196 ppm hydrocyanic acid for 4 months (Maner & Gomez, in *Chronic Cassava Toxicity: Proceedings of an Interdisciplinary Workshop*, London, 1973: Int. Devel. Res. Centre Monogr. IDRC-010e, 1973, p. 113). However, the same authors showed that 480 ppm cyanide (as KCN) reduced rat growth and feed consumption, and at 960 ppm some

deaths occurred. The present study was designed to investigate whether the toxic effects of cyanide, fed in a nutritionally adequate diet, could be transmitted to the offspring.

As a cassava diet naturally high in cyanide could not be obtained, potassium cyanide was added to a nutritionally adequate diet containing 60·3% cassava meal to produce a dietary level of 500 ppm cyanide. When fed to female rats throughout gestation and/or lactation this diet had no statistically significant effect on the body-weight gain, feed consumption or relative liver and kidney weights of the adults, on litter size, or on pup birth weight, weaning weight or mortality, despite a marked rise in serum thiocyanate in both the dams and their pups. However, the same diet fed to the offspring for 28 days during the postweaning growth phase significantly reduced feed consumption, growth rate and protein efficiency ratio, although serum thiocyanate levels were no higher than in the treated dams. Serum thiocyanate levels in the offspring were not affected by previous treatment of the mothers, whereas protein efficiency ratios were somewhat reduced even in the untreated offspring of dams fed the test diet during gestation and/or lactation. Rhodanase activity in liver and kidney was unaffected by treatment at any stage.

The increase in serum thiocyanate in both mothers and offspring indicated that some organic sulphur had been used in detoxifying cyanide, and the authors suggest that use of the sulphur-containing amino acids for this purpose may have caused the poorer growth rate of postweaning rats. There was a slight reduction in protein efficiency ratio even in the untreated offspring of treated mothers, suggesting that the effects of exposure *in utero* or during lactation may be manifested only after weaning. As the effects of inorganic cyanide and cyanogenic glycosides may not be identical, further studies with pure linamarin are considered necessary.

Carcinogenicity of stored bracken

Kawai, T., Takanashi, H., Nakayama, M., Mori, H. & Hirono, I. (1981). Effects of storage on carcinogenic activity of bracken fern. *Cancer Lett.* **12,** 29.

Exhaustive studies have failed to identify the substance responsible for the carcinogenic activity of the bracken fern (*Pteridium aquilinum*) (*Cited in F.C.T.* 1980, **18,** 311) but certain of its properties have been characterized including its major location in the plant (*ibid* 1974, **12,** 285), its solubility (*ibid* 1971, **9,** 920; *ibid* 1979, **17,** 421) and the inhibition of its activity by various factors (*ibid* 1978, **16,** 506). In an attempt to discover more about the nature of the active agent, the impact of duration and conditions of storage on the carcinogenic action of dried bracken fern have now been examined.

Twenty rats of both sexes received a diet containing fresh ground dehydrated bracken initially at a ratio of 1:2 (bracken:diet). However because of the unpalatability of the bracken diet, after 72 days the ratio was reduced to 1:4 for 76 days and the basal diet was fed on 22 days. Diet containing bracken powder that had been stored for 1 or 2 yr in a shaded container at a temperature of 4°C or at room temperature was fed for 90 days to groups of 17–18 rats of both sexes. The stored bracken was mixed with the diet at a ratio of 1:2 (bracken:diet).

Nineteen of the 20 rats given fresh bracken survived beyond 7 months after the start of the experiment and 15 developed ileal adenomas, adenocarcinomas or fibrosarcomas. The rats with intestinal tumours had a mean of 4·3 tumours each, mainly confined to the anal 20 cm of the ileum. One rectal and three caecal adenomas were also found. All but one of the animals given bracken stored for 1 yr survived beyond 7 months. Twelve of the 17 surviving animals given bracken stored at 4°C and ten of the 16 surviving animals given bracken stored at room temperature developed ileal adenomas, adenocarcinomas or fibrosarcomas. The mean number of tumours/tumour-bearing rat was 2·3 (bracken stored at 4°C) or 1·7 (bracken stored at room temperature). One rat in this group had a caecal tumour. All of the animals given bracken that had been stored for 2 hr survived beyond 300 days but ileal tumours (1·2–1·3 tumours/animal) were observed in four of the 18 rats given bracken stored at 4°C and two of the 18 rats given bracken stored at room temperature. Caecal tumours were found in five animals of the latter group. The incidence of tumours in the groups given bracken stored for 2 yr was significantly less than that in the animals given fresh bracken. In general the latent periods of intestinal tumours in the groups given stored bracken were longer than those of the groups given fresh bracken. Several rats in the treatment groups showed transitional cell hyperplasia of the urinary bladder but no bladder tumours were detected. The only tumours appearing in the three control groups were one pituitary adenoma and one adrenal phaeochromocytoma.

Thus the carcinogenic activity of the bracken fern seems to be reduced by long-term keeping, independent of ambient storage temperature. The results also imply that attempts to isolate the active principle would be best made on fresh fern.

Bracken and the gut microflora

Sumi, Y., Hirono, I., Hosaka, S., Ueno, I. & Miyakawa, M. (1981). Tumor induction in germ-free rats fed bracken (*Pteridium aquilinum*). *Cancer Res.* **41,** 250.

Now a familiar topic with regular readers, the bracken fern (*Pteridium aquilinum*) carcinogenicity issue was first raised within these pages in 1966 (*Cited in F.C.T.* 1966, **4,** 358). Despite intensive efforts, the identity of the active principle involved has remained undiscovered although characterization of certain of its features has been achieved (see above). The current study continues the investigations of this naturally-occurring carcinogen.

Ten germ-free and 16 conventional rats weighing 80–90 g at the start of treatment were fed a sterilized diet comprising one part milled dried bracken fern to two parts basal diet, for the remainder of their lives. The germ-free rats were maintained in a plastic isolator and their germ-free status was checked fortnightly. All of the animals were autopsied when they

died or were killed (when moribund) and various organs, including the entire gastro-intestinal tract were examined for tumours.

Tumours developed in 90% of the germ-free rats and 94% of the conventional rats with average latent periods of 11·7 and 13·7 months respectively. The average numbers of tumours were 4·6/germ-free rat and 4·4/conventional rat. Apart from three germ-free rats with caecal tumours all of the intestinal tumours were located in the distal part of the ileum. Of the germ-free rats six had ileal adenomas, three had caecal adenomas, one had an adrenocortical adenoma and nine had sarcomas. Of the conventional rats eight had ileal adenomas, ten had iliac adenocarcinomas, one had a papilloma of the urinary bladder, two had adrenocortical adenomas and five had iliac sarcomas. The sarcomas of germ-free rats were larger, more numerous and more rapidly growing than those of conventional rats. The non-intestinal tumours were one adrenocortical adenoma in a germ-free rat and two adrenocortical adenomas and one bladder tumour in conventional rats.

There was no apparent difference between germ-free and conventional rats in tumour incidence although the absence of gut microflora was associated with a shorter latent period, increased susceptibility to sarcomas and reduced susceptibility to adenocarcinomas. The authors suggest that these differences were related to some degradation of the bracken carcinogen by the gut microflora in conventional rats.

[In view of the small numbers of animals involved and the absence of a control group these results can only be regarded as a preliminary indication.]

Laboratory animals—turning the tables?

Cockcroft, A., Edwards, J., McCarthy, P. & Andersson, N. (1981). Allergy in laboratory animal workers. *Lancet* **I**, 827.

Nearly 32,000 people work with laboratory animals in the UK and symptoms of allergy, particularly rhinitis and asthma, seem to be quite common. Many more people are exposed to animals in different situations and there have been a number of reports of allergic reactions (including one to elephants!).

Cockcroft *et al.* have investigated the extent and nature of the animal allergy problem in three MRC establishments. A questionnaire detailing the amount of past and present animal contact and noting any history of respiratory symptoms, asthma or hay fever was completed by 213 volunteers. Of these, 179 were classed as 'handlers' who were engaged in full-time animal husbandry, or 'users', those who used animals experimentally. Subjects were also asked about symptoms from animal contact. Skin-prick tests were used to establish allergic responses and atopic individuals were identified by a positive response to one or more of six common environmental allergens such as tree pollen and house dust. Reactions to skin-prick tests with serum and urine extracts from rats, mice, guinea-pigs, rabbits and sheep were assessed. In addition, sera from the subjects were tested by double gel diffusion against antigens from the five animal species at 1 mg/ml and the enzyme-linked immunosorbent assay technique was used on the sera.

Of the 179 exposed subjects 31% (55) gave a history of asthma and/or hay fever and 39% (70) were classed as atopic. Among the small group of non-exposed subjects 35% were classed as atopic. Forty-nine, (27%) of the exposed subjects had symptoms relating to animal contact, most commonly rhinitis but sometimes also asthma, and rashes and local urticaria often followed animal scratches or contact with urine or paws. The symptoms in most cases were relatively minor but they occasionally prevented further contact with animals. There was no overall tendency for the more heavily exposed handlers to have more allergic symptoms than the users although handlers did develop symptoms more quickly than users. There was no association between skin-test atopic status and symptoms from animal contact with the exception of asthma. Subjects with a previous history of asthma were more likely to develop animal-related asthma but no more likely to develop other symptoms. Smoking history was not related to symptoms from animal contact. Of the 179 exposed subjects 29 had a positive reaction to at least one of the animal extracts after 15 min and this skin reactivity was positively associated with a history of symptoms from animal contact although most of this association was due to the strong association between skin reactivity and asthma from animal contact. Skin reactivity to animal extracts was also associated with skin-test atopic status. No precipitins were detected in the sera of subjects by gel diffusion against the animal extracts. The enzyme-linked immunosorbent assay showed no significant differences in IgG levels between the various groups.

The authors considered that their results may have underestimated the total number of allergy sufferers especially since many of the workers had only recently joined the units and may not have experienced symptoms, whilst the worst cases may have given up working with animals completely. This second factor may have had quite a significant effect since Lutsky & Neuman (*Ann. Allergy* 1975, **35**, 201) found that 28% of the workers who were allergic to animal dander in 39 establishments in the USA subsequently changed jobs or avoided contact with specific animal species.

Only five individuals showed skin reactivity in the absence of symptoms suggesting that skin tests are unlikely to be useful as a prescreen for people likely to develop symptoms. Many workers who had experienced animal-associated rhinitis gave negative results in the skin tests. In addition, it was not possible to predict susceptible individuals on the basis of their response to the standard battery of allergens although atopic individuals were more likely to develop animal-related asthma. The authors considered it unlikely that attempts to screen new entrants to identify potential sufferers would be successful and suggested that future efforts should mainly be directed to reducing exposure.

Methyl chloride and behaviour

Putz-Anderson, V., Setzer, J. V., Croxton, J. S. & Phipps, F. C. (1981). Methyl chloride and diazepam

effects on performance. *Scand. J. Work Envir. Hlth* **7**, 8.

It has been reported that methyl chloride produces subtle but quantifiable behavioural effects at levels below the current TLV of 100 ppm. Repko *et al.* (NIOSH Technical Information Publ. No. 77–125, 1976) found that 122 workers exposed to a mean concentration of 34 ppm methyl chloride showed impaired performance on cognitive time-sharing tasks and increased finger tremor although there was no detectable increase in the incidence of abnormal neurological symptoms compared with controls. However Hake *et al.* (*Toxic appl. Pharmac.* 1977, **41**, 198) exposed volunteers repetitively on a daily basis to methyl chloride concentrations of 20, 100 and 150 ppm for periods of 1, 3 or 7·5 hr with no deleterious neurological or behavioural effects. Putz-Anderson *et al.* (cited above) sought to clarify the behavioural effects of acute exposure to permissible levels of methyl chloride and to investigate the effects of concomitant administration of diazepam (valium). It was thought possible that any behavioural effects of methyl chloride might be aggravated by a central nervous system depressant such as diazepam.

Volunteers aged 18–32 were randomly assigned to six groups, four groups of 12 and two groups of eight. Each volunteer took a capsule either of 10 mg diazepam or of a placebo 30 min before exposure for 3 hr to 0, 100 or 200 ppm methyl chloride in the air. Only professed non-users of diazepam were asked to participate and they were all required to abstain from caffeine, alcohol and medical treatment for 24 hr before exposure. The three behavioural tests of visual vigilance and dual-task and time-discriminating abilities were used to assess different aspects of attention or alertness, during the 2 hr prior to treatment and during the 3-hr treatment period. Alveolar breath samples were taken hourly and blood samples were taken just before exposure and about 90 min after the start of exposure.

Diazepam produced a significant effect in all of the behavioural tests resulting in an average decline of 10·1% in performance compared with controls. Methyl chloride treatment at the high dose resulted in a marginally significant (4%) decline in performance. (The low-dose methyl chloride groups were not included in the statistical analyses because of their small group sizes—eight/group.) The effect of the two treatments combined was additive but not synergistic producing a total reduction in performance of 13·5%. As expected breath and blood levels of methyl chloride were highly correlated but there were considerable inter-individual variations in body burden within groups exposed to the same level. Hake *et al.* (*loc. cit.*) had found a similarly high degree of variation between individuals.

[Although this study indicates that acute exposure to permissible levels of methyl chloride does not have marked behavioural effects the usefulness of such results in determining the effects of chronic exposure in the workplace is not clear-cut. It may also be relevant that the onset of neurological symptoms in severe methyl chloride poisoning is delayed possibly for 24–48 hr (Repko & Lasley, *CRC Crit. Rev. Toxicol.* 1979, **6** (4), 283).]

PVC-inhaling rats again

Richards, R. J., Rose, F. A., Tetley, T. D., Cobb, L. M. & Hardy, C. J. (1981). Effects in the rat of inhaling PVC dust at the nuisance dust level (10 mg/m^3). *Archs envir. Hlth* **36**, 14.

Studies to assess the degree of hazard arising from the inhalation of polyvinyl chloride (PVC) dusts have yielded inconclusive results. Suspension homopolymer PVC dust samples have proved to have little if any haemolytic action *in vitro*, while paste (emulsion) polymer PVC dusts containing surfactants produce extensive red blood corpuscle (RBC) damage (Richards *et al. Nature, Lond.* 1975, **256**, 664; *idem ibid* 1976, **260**, 53). The surfactant was considered responsible for this activity (*idem ibid* 1976, **260**, 53), and for the toxicity to rat peritoneal macrophages *in vitro* (*Cited in F.C.T.* 1981, **19**, 277). In rats single intratracheal instillations of 2 or 20 mg suspension or paste polymer PVC dust produced no progressive fibrogenic response (*ibid* 1980, **19**, 277), but single intratracheal doses of 25 mg (unspecified) PVC dust have been shown to induce biochemical and histopathological changes in rat lung tissue (Agarwal, *Envir. Res.* 1978, **16**, 333). An earlier study had produced evidence of granulomatous foci in the lungs of guinea-pigs exposed continuously for 2–7 months in a PVC bagging plant (Frongia *et al. Medna Lav.* 1974, **65**, 321), and there have been isolated reports of pneumoconiosis in PVC workers (e.g. Arnaud *et al. Thorax* 1978, **33**, 19; Szende *et al. Medna Lav.* 1970, **61**, 433). However, a further study of 509 men who worked in a PVC-coated-fabrics and wall-coverings factory, some of whom had been exposed to PVC dust for over 15 yr, showed no significant deterioration in lung function (*Cited in F.C.T.* 1981, **19**, 512).

Richards *et al.* (cited above) carried out a study to determine whether the inhalation of a paste polymer PVC-7 (containing the detergent sodium dodecyl sulphate) at 'nuisance' dust levels (10 mg/m^3; Guidance Note EH 15/77, Health and Safety Executive, London, 1977) would produce any alteration in the lungs of experimental animals.

Eighty 10-wk-old female albino Sprague–Dawley CD rats were randomly allocated to either a control group or a PVC-exposed group (40 rats in each). The PVC-treated animals were exposed to a mean concentration of 10·6 mg dust/m^3 for 6 hr/day, 5 days/wk for up to 15 wk. Groups of 10 exposed rats were examined and compared with control animals at 3, 9 and 15 wk. One group of animals exposed for 15 wk was maintained for a further 15 wk with no further contact with PVC and then examined with a non-exposed control group also maintained during this period.

The lungs showed small, randomly scattered lesions after 15 wk of exposure. These lesions were characterized by hypercellularity of the interstitium of the alveolar walls in areas adjacent to macrophage aggregates containing numerous PVC particles. While the lesions persisted 15 wk after the cessation of exposure, there were only minimal increases in collagen and reticular fibre formation, and no evidence of an extensive fibrotic reaction. Relatively few biochemical changes were detected at any exposure period. Three significant changes in rats exposed to PVC were (1)

elevated pulmonary surfactant, (2) depressed lung protein 'synthesis' at 9 wk, (3) elevated lung acid RNAase activity at 15 wk.

The results suggest that even at 'nuisance' dust levels, PVC has a demonstrable biological activity. In relative terms its activity is low; in previous inhalation studies on similar levels of chrysotile and amosite asbestos and fibre glass elevated free-cell numbers, increased free-cell and/or lung enzyme activity, and a large increase in pulmonary surfactant were found.

[Exposure was carried out in a 240-litre perspex chamber. It is not clear whether any anti-static precautions were taken. In addition, the authors do not mention whether the uniformity of the atmosphere in the chamber was checked by sampling from various points in the chamber.]

Vinyl chloride and chromosomes

Anderson, D., Richardson, C. R., Weight, T. M., Purchase, I. F. H. & Adams, W. G. F. (1980). Chromosomal analyses in vinyl chloride exposed workers. Results from analysis 18 and 42 months after an initial sampling. *Mutation Res.* **79**, 151.

In an earlier study (*Cited in F.C.T.* 1980, **18**, 200) a group of 57 workers exposed to vinyl chloride (VC) were reported to have an increased incidence of chromosomal abnormalities in their lymphocytes compared with 24 control workers. Unfortunately exposure data available at that time for those workers were not accurate but since then there have been significant reductions in vinyl chloride exposure. Anderson *et al.* have therefore re-examined the same group of workers to see whether the reduced exposure levels have lowered the incidence of chromosomal abnormalities.

In the previous study the workers were examined in July 1971. Of the original 57 exposed workers, 21 were re-examined in January 1976 and 23 were further examined in January 1978. Each subject gave details of his medical and occupational history. The second sampling (January 1976) included ten autoclave workers, eight men involved in VC manufacture or maintenance of VC and polyvinyl chloride (PVC) plants, three former PVC production workers who were no longer exposed to VC and six controls not exposed to VC. The third sampling (January 1978) included six autoclave workers, ten men involved in VC manufacture or maintenance of VC and PVC plants, seven former PVC production workers and eight controls not exposed to VC.

Lymphocyte cultures were prepared from blood samples taken from the workers and the chromosomal abnormalities observed in 48- and 72-hr cultures were classified according to type. At the January 1976 sampling 11 of the men had colds but there was no evidence to suggest that this caused an increase in any type of abnormality. In 1976 the autoclave workers showed a statistically significantly larger increase over 1974 values than did controls in B cells (containing chromatid gaps, chromosome gaps, chromatid breaks and chromatid interchanges), total abnormalities and abnormalities excluding gaps. The three men who had left PVC production showed a significant decrease in these sorts of chromosome damage compared with the overall trend. The same pattern was seen for other types of chromosome damage but the differences were not statistically significant. There were no significant differences when the 1974 and 1976 samplings were compared for the non-autoclave workers and the controls. Considering the 1976 samples alone, the exposed workers all had higher percentages of all types of abnormalities than did the control group. At the third sampling (January 1978) the percentage of cells with chromosomal aberrations tended to decrease compared with previous samplings. Exposed workers showed decreases in the mean numbers of certain types of abnormalities and the results for these groups were comparable to those for controls and former workers who were no longer exposed. When the results of the 1978 sampling were considered alone there was no significant difference between exposed workers and controls. The data for the 12 individuals who were present at all three samplings showed a decrease in chromosome damage in all groups (including controls) at the 1978 sampling compared with previous samplings. Since the slides from the third sampling had been read by someone different, some of them were re-analysed by the person who had done the original readings and this produced even lower values suggesting that observer bias was probably not responsible for the decrease.

The average exposure to VC in PVC plants had been steadily reduced from 300–400 ppm in the 1960's to 150 ppm by mid 1973, 50 ppm in mid 1974, 5 ppm in 1975 and below 5 ppm since then. The normal levels of chromosome damage found in workers in 1978 suggested that the reduction in exposure to VC had allowed chromosomal abnormalities to return to normal. The increase in some categories of abnormalities in 1976 may have been due to seasonal variations.

Little hazard from barium in drinking-water

Tardiff, R. G., Robinson, M. & Ulmer, N. S. (1980). Subchronic oral toxicity of BaCl2 in rats. *J. envir. Path. Toxicol.* **4**, (5–6), 267.

When drinking-water containing 5 ppm barium (Ba) as the acetate was given to rats and mice throughout their lives, it produced little in the way of adverse effects. Lifespan was slightly reduced in the mice and female rats, there was some proteinuria in male rats, and the female rats were slightly overweight towards the end of the study (*Cited in F.C.T.* 1976, **14**, 217 & 360). The acute toxicity of Ba salts varies with solubility, and Ba sulphate (which is practically insoluble in water) was only lethal to rats in doses in 25–40% of their body weight (*ibid* 1967, **5**, 245). Ba occurs in all human tissues, with over 90% of the body burden in bone, and about 6% of that ingested is derived from natural waters (*ibid* 1974, **12**, 278). The US interim primary drinking-water regulations set a limit of 1 mg/litre for Ba (*Federal Register* 1975, **40**, 11990; *ibid* 1975, **40**, 59566) but this is derived from the TLV for industrial exposure rather than from chronic feeding studies (Calabrese, *Med. Hypotheses* 1979, **5**, 653). Moreover several potable water supplies

contain Ba in excess of this level (*idem, J. envir. Hlth* 1978, **39,** 366).

To define better the subacute oral toxicity of Ba, Tardiff *et al.* (cited above) gave drinking-water containing 10, 50 or 250 mg Ba/litre to rats for 4, 8 or 13 wk. The Ba was in the form of the chloride, which was considered representative of soluble Ba in potable water. The highest level was equivalent to daily doses of 64·2 mg/kg body weight in males and 68·3 mg/kg in females at the start of the study and to 27·5 mg/kg in males and 35·5 mg/kg in females at the end, and was initially equivalent to about one third of the LD_{50} value (which was found to be 220 mg Ba/kg in weanling rats and 132 mg Ba/kg in adult rats). Levels present in the diet contributed a background daily dose of about 0·5 μg Ba/kg body weight. No adverse effects were observed on food consumption, mortality, clinical signs, body weight, haematology, serum enzyme activities, serum sodium, potassium or calcium, or the weight or histopathology of the liver, kidneys, spleen, heart, brain, muscle or femur. The only effects were a decrease in water consumption at the highest dose level, and a slight decrease in relative adrenal weight in males after 8 wk at 50 and 250 ppm and in females after 13 wk at all levels. However, in females after 8 wk adrenal weight was significantly increased at the highest level. Analysis of liver, bone, muscle and heart revealed barium concentrations that increased with increasing dose, but not with time. Bone contained approximately 50 times more barium than the other tissues in controls, and 200 times more than other tissues in the highest dose group.

The effect on adrenal weight was thought to be correlated with a previously demonstrated activation of catecholamine secretion from the adrenal medulla by Ba (Douglas & Rubin, *Nature, Lond.* 1964, **203,** 305; Shanbaky *et al. Toxic appl. Pharmac.* 1978, **44,** 99). Intravenously administered Ba is rapidly removed from the blood (Chou & Chin, *Chin. Med. J.* 1943, **61,** 313), and it is suggested that the levels in this study (despite being up to 1/3 the LD_{50} value) were insufficient to overwhelm the high rate of elimination and of distribution to bone.

Inorganic tin and bone strength

Ogoshi, K., Kurumatani, N., Aoki, Y., Moriyama, T. & Nanzai, Y. (1981). Decrease in compressive strength of the femoral bone in rats administered stannous chloride for a short period. *Toxic. appl. Pharmac.* **58,** 331.

Previous studies have clearly demonstrated the ability of inorganic tin to interfere with calcium metabolism in the rat. Effects noted in the kidney, following ip doses of tin chloride, including an increase in the cortical level of calcium associated with decreased serum calcium concentrations (Yamamoto *et al. J. Toxicol. envir. Hlth* 1976, **1,** 749), were subsequently found to be related to elevated calcium binding activities of the soluble fraction of the renal cortex (Yamaguchi *et al. ibid* 1977, **3,** 413) and partly caused by inhibition of calcium efflux from the cells (Yamaguchi, *Toxicology Lett.* 1979, **4,** 45). Oral doses of inorganic tin have also been shown to inhibit the intestinal absorption of calcium (Yamaguchi *et al. Toxic. appl. Pharmac.* 1979, **47,** 441) and to increase its biliary excretion (Yamamoto, *ibid.* 1978, **45,** 611).

The depletion of calcium levels observed in the femoral bone following tin treatment has been attributed to the increased mobilization of calcium from bone into blood as a result of a parathyroid-hormone-induced increase in femoral acid phosphatase activity (Yamaguchi *et al. Toxicology Lett.* 1979, **3,** 7; Yamaguchi & Okada, *ibid* 1979, **4,** 39). The effect of orally administered stannous chloride on the mechanical strength of the bones of rats has now been investigated.

Groups of 25–30 young male rats fed on diets containing 52·4 ppm tin were permitted free access to water containing 0, 50, 150, 300 or 600 ppm tin (as stannous chloride) for 4 wk. On day 29 the rats were killed and the entire femurs of each animal were dissected out, cleaned of soft tissue, and both ends were embedded in hollow cylinders with epoxy resin. After storage at 4°C at a high humidity for 48 hr to stiffen the resin, compression tests were carried out with the mounted bones in an Instron apparatus, loaded in the direction of the long axis of the bone at a deformation rate of 0·5 mm/min. The load-deformation curve was recorded for each bone.

The compressive strength of the femoral distal epiphysis was reduced at the two highest treatment levels and this may be due to the decreased calcium content of the bone. However, since no change in the diaphyseal compressive strength was noted, the authors considered that the decreased calcium content of the bone could not be the only factor responsible for the loss of bone strength. They concluded that further investigation into this aspect of inorganic-tin toxicity is required, particularly since the safe limit of tin exposure may be lower than that currently accepted.

Toxicity and mutagenicity of *N*-chloropiperidine

Bempong, M. A. & Scully, F. E., Jr (1980). Mutagenic activity of *N*-chloropiperidine. *J. envir. Path. Toxicol.* **4** (2,3), 345.

The mixtures of non-volatile organic chemicals that are present in some samples of chlorinated drinking-water have been demonstrated to be mutagenic in the Ames *Salmonella typhimurium* plate test (*Cited in F.C.T.* 1981, **19,** 136). Organic *N*-chloramines are among the chlorination products of water disinfection and the authors cited above have studied the toxicity and mutagenicity of one such compound, *N*-chloropiperidine (NCP).

The LD_{50} values for NCP and piperidine given intraperitoneally (ip) were determined in 4–6-wk-old mice. The doses given to groups of ten male and ten female mice were 50, 100, 200, 300 and 400 mg/kg body weight. The LD_{50} values of both compounds varied greatly depending upon the age of the solution used; that of piperidine increased from about 60 to about 300 mg/kg body weight as storage time increased from 1 to 5 days whereas that of NCP decreased from about 300 to about 100 mg/kg body weight as storage time increased from 1 to 5 days. The authors were puzzled by these changing toxicities

which were presumably caused by a chemical reaction mediated by water. NCP showed significant mutagenic activity in the absence of microsomal activation in *Salmonella typhimurium* strains TA100 and TA1536. Faecal material, urine and peritoneal fluid from mice injected ip with 50 or 100 mg NCP/kg body weight produced histidine revertants in a plate incorporation assay with *S. typhimurium* strain TA100 and to a lesser extent strain TA1535. The mutagenic activity of the peritoneal fluid was greatest and that of the urine was least. The number of revertants induced by the peritoneal fluid and the faecal material exceeded that induced by similar material from untreated mice. In the plate incorporation assay with *S. typhimurium* strains TA100 and TA1535 without metabolic activation, the greatest mutagenic activity of NCP was obtained at a level of 64 μg/plate. At levels above this the toxic effects of the compound took over. In the plate incorporation assay the peritoneal fluid from NCP-treated mice produced more revertants than were produced by direct incorporation of NCP. Possible explanations for this phenomenon might be that some of the injected material was converted to more active mutagens or that small amounts of the compound were detoxified *in vivo*.

NCP would seem to be mutagenic in *S. typhimurium* but further work is needed to clarify the toxicity, mutagenicity and metabolism of this compound.

Not only hard on resins

Benjamin, T., Evarts, R. P., Reddy, T. V. & Weisburger, E. K. (1981). Effect of 2,2'-diaminodiphenyldisulfide, a resin hardener, on rats. *J. Toxicol. envir. Hlth* 7, 69.

The selection of 2,2'-diaminodiphenyldisulphide (DDDS) as a possible non-carcinogenic alternative to the resin hardener, 4,4'-methylenebis-(2-chloroaniline) (*Cited in F.C.T.* 1976, **14**, 217) was based on a consideration of structure–activity relationships. The present study was carried out to evaluate whether, in fact, a suitable choice had been made and whether the compound's development for use in resins should proceed.

Three groups of 15 rats received diets containing 1% DDDS (the maximum tolerated dose established in preliminary investigations) for 13 wk, followed by control diet for 72 wk (group I), 0·5% DDDS for 85 wk (group II), or the control diet only. Two animals from each group were killed at wk 20 to check for early lesions. The remainder were autopsied after 85 wk and the liver, lungs, kidneys, spleen and gastrointestinal tract, plus any other tissues that appeared abnormal, were removed for further examination. In view of the lipidosis induced by structurally similar compounds, lipid determinations were carried out on the liver, spleen and kidney.

Proliferation of the bile ducts in the liver occurred in all three groups, but only in the treated rats was this accompanied by centrilobular necrotic changes (group I) or areas of clear cells or basophilic cells (group II). One well-defined hepatocellular adenocarcinoma and one neoplastic liver nodule were also identified in group II. The kidneys of both treated and control rats showed dilated tubules contained protein casts and lined with atrophic epithelium, cortical tubules lined by enlarged cells containing basophilic cytoplasm and large nuceli, haemosiderin pigmentation and interstitial-cell infiltration, but these findings were more marked in the treated animals. The most pronounced effects of treatment were seen in the forestomach. Acanthosis and hyperkeratosis, formation of keratin pearls and penetration of basal cells through the muscularis mucosae into the submucosa, or their downgrowth to form oval masses in the lamina propria, occurred only in the treated groups (more prominently in group II).

Total liver phospholipid, phosphatidyl ethanolamine and cholesterol concentrations were significantly elevated in both treatment groups, but the levels of phosphatidyl choline (in group II) and of triglycerides were depressed. In the kidney and spleen the amounts of total phospholipids, phosphatidyl choline, cholesterol and phosphatidyl ethanolamine were significantly increased in the treated animals while triglyceride levels were reduced.

Modified Ames tests in two strains of *Salmonella typhimurium*, with and without metabolic activation of the compound, were negative even at high concentrations of DDDS (1000 μg/plate).

Although the 85-wk study may not have been sufficiently prolonged to reveal the full carcinogenic activity of DDDS, it has demonstrated the compound's hyperplastic potential and its unsuitability for use as a substitute for methylenebis-(2-chloroaniline).

[This discrepancy between the expected effect of a compound designed according to structure–activity principles and its actual properties *in vivo* underlines the pitfalls that are likely to be encountered before this promising approach reaches its full potential.]

A factor in xylene embryotoxicity?

Ungváry, G., Varga, B., Horváth, E., Tátrai, E. & Folly, G. (1981). Study on the role of maternal sex steroid production and metabolism in the embryotoxicity of *para*-xylene. *Toxicology* **19**, 263.

The widely used industrial and laboratory chemical, xylene, has been shown to have embryotoxic effects in rats (*Cited in F.C.T.* 1981, **19**, 796). In the present study, which aimed to identify the mechanism by which this occurs, the effects of *p*-xylene on maternal sex steroid production and metabolism in the pregnant rat, have been examined.

Groups of 20 pregnant rats were exposed to either uncontaminated air for 48 hr, or air containing 3000 mg *p*-xylene/m^3 for 24 or 48 hr on day 10, or days 9 and 10 of gestation (depending on the duration of treatment). Two hours after the end of exposure the animals were anaesthetized and the uterus and ovaries were exposed for the cannulation of one of the utero-ovarian veins and the collection of blood samples. Blood was collected separately from the ipsilateral ovary and uterine horn. Whilst blood flow in the ovary showed a decreasing trend with increasing *p*-xylene exposure, no effects on uterine blood flow were apparent. The levels of ovarian progesterone and 17 β-oestradiol were not significantly changed by

treatment but a significant depression of these hormone levels was seen in blood taken from the femoral and uterine veins of rats exposed to xylene for 48 hr. Removal and weighing of the uteri revealed a reduction in foetal weight in animals from the 48-hr exposure group, but no marked lethality was observed and the placentas were found to be intact both by gross inspection and routine histological examination.

Although information on the development of arterio-venous shunts that might develop under the influence of the toxic agent or on the flow of nutrients to the uterus was not available the authors conclude from these investigations that uterine blood flow and the ovarian secretion of hormones were not affected by *p*-xylene. It was suggested, however, that the reduction in the levels of maternal sex steroids in blood from the uterine and femoral veins resulted from their enhanced biotransformation due to stimulation of the hepatic mixed-function-oxidase system by *p*-xylene treatment and that these decreased peripheral hormone levels may play a part in the embryotoxicity of *p*-xylene.

The safety of methyl amyl ketone

Lynch, D. W., Lewis, T. R., Moorman, W. J., Plotnick, H. B., Schuler, R. L., Smallwood, A. W. & Kommineni, C. (1981). Inhalation toxicity of methyl *n*-amyl ketone (2-heptanone) in rats and monkeys. *Toxic. appl. Pharmac.* **58,** 341.

Peripheral neuropathy has resulted from exposure to methyl *n*-butyl ketone (2-hexanone) and its metabolites 2,5-hexanediol and 2,5-hexanedione (*Cited in F.C.T.* 1981, **19,** 133). However, there has been little evidence of this or other severe toxic effects from methyl *n*-amyl ketone (2-heptanone; MAK). When fed to rats at levels of up to 500 mg/kg body weight/day for 13 wk MAK produced only increases in urinary cell excretion and in liver and kidney weights at high dose levels (Gaunt *et al. Fd Cosmet. Toxicol.* 1972, **10,** 625) and there was a similar lack of neurotoxicity in rats given drinking-water containing 0·5% MAK for 14 wk (Spencer *et al. Toxic. appl. Pharmac.* 1978, **44,** 17). In rats a single intraperitoneal injection of 37 mg/kg or more, significantly reduced the fixed-interval response rate, and a similar but non-significant effect was seen after inhalation of 1575–1900 ppm for 8 hr (Anger *et al. ibid* 1979, **49,** 407). However, no neurophysiological effects were seen in male rats and monkeys inhaling mean levels of 131 or 1025 ppm MAK, 6 hr/day, 5 days/wk for up to 10 months (Johnson *et al. Am. ind. Hyg. Ass. J.* 1978, **39,** 866; *idem, J. envir. Path. Toxicol.* 1979, **2** (5), 113). Details of other observations made in the last study have now been reported.

In both the rats (50 males per group) and monkeys (eight males per group) there were no effects on body weights or on the histopathology of lungs, liver, heart, spleen, kidneys, adrenals, pancreas, testes, brain or the tibial branch of the sciatic nerve. Comprehensive cardiopulmonary studies on the monkeys after 6 months revealed values that were consistently higher than those of the controls at both exposure levels in five of the 21 tests conducted, but the changes were neither statistically significant nor dose-related. Electrocardiographic examination also revealed no exposure-related effects. In clinical chemistry studies on monkey blood after 1, 3 and 6 months, a few parameters (such as serum inorganic phosphate) were significantly different from controls in one group at one sampling period, but overall there were no dose-related alterations.

Pentobarbital sleeping time in the rats at the end of the study was unaffected, suggesting a lack of microsomal enzyme induction. MAK was detected in both the serum and urine of rats and monkeys at both exposure levels, and low levels of methyl *n*-amyl alcohol were found in monkey serum and urine at 1025 ppm. Chromatographic peaks also suggested the presence of 2,6-heptanedione and 2-keto-6-hydroxyheptane, but these were not confirmed. In rats exposed to ^{14}C-labelled MAK at 132 ppm for 6 hr or given an ip injection of 10 mg/kg, urinary excretion peaked at 12 hr and accounted for 25% of the dose, whereas just over 1% had appeared in the faeces after 72 hr. Regardless of exposure route the liver contained the highest level of radioactivity, followed in general by kidney, pancreas and lung. Only low levels were found in the brain, and levels in the sciatic nerves were below the limit of detection. Prior exposure to unlabelled MAK for 6 months did not alter the pattern of distribution.

[The TLV currently recommended by ACGIH for MAK is only 100 ppm (465 mg/m^3) and it is proposed to reduce this even further, to 50 ppm (*TLVs. Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1980.* ACGIH, Cincinnati, OH, 1980). The present study indicates that the existing TLV provides a fair margin of safety.]

Urinary hexane metabolites

Perbellini, L., Brugnone, F. & Faggionato, G. (1981). Urinary excretion of the metabolites of n-hexane and its isomers during occupational exposure. *Br. J. ind. Med.* **38,** 20.

It is possible to determine levels of exposure to commercial hexane (containing *n*-hexane, 2-methylpentane and 3-methylpentane) by measuring environmental levels, alveolar-air levels and by blood tests (Brugnone *et al. Int. Archs occup. envir. Hlth* 1978, **42,** 51; *idem ibid* 1979, **42,** 355) but not by direct measurement of hexane in the urine. However, metabolites of *n*-hexane, and of 2- and 3-methylpentane have been found in the urine of shoe-factory workers and thus an attempt has been made to determine whether a correlation exists between environmental commercial hexane exposure and metabolite excretion.

Individual environmental solvent levels were measured in the breathing zone of workers at five different shoe factories during the afternoon shift. The environmental air was found to contain *n*-hexane, and 2- and 3-methylpentane. Very low levels of methylcyclopentane, cyclohexane, 2,3-dimethylbutane, acetone, dichloromethane and trichloroethylene were also found occasionally. Environmental exposure to *n*-hexane ranged from 32 to 500 mg/m^3 with a mean of

182 mg/m^3; exposure to 2-methylpentane ranged from 11 to 250 mg/m^3 with a mean of 77 mg/m^3 and exposure to 3-methylpentane ranged from 10 to 204 mg/m^3 with a mean of 63 mg/m^3. At the end of the afternoon shift urine samples were collected from 41 workers and analysed for *n*-hexane and 2- and 3-methylpentane metabolites. The urine samples were found to contain 2,5-hexanedione, 2-hexanol, 2,5-dimethylfuran and γ-valerolactone. The percentage concentrations of these *n*-hexane metabolites varied widely but in most cases 2,5-hexanedione was the main metabolite (mean concentration 5·4 mg/litre). The urinary concentration of 2,5-hexanedione correlated with that of 2-hexanol and of 2,5-dimethylfuran but no other such correlations were found among the *n*-hexane metabolites. The excretion concentrations of the 2- and 3-methylpentane metabolites, 2- and 3-methyl-2-pentanol, were found to be correlated with the environmental concentrations of their parent compounds. All of the *n*-hexane metabolites considered together were well correlated with environmental *n*-hexane concentrations. 2-Hexanol and 2,5-hexanedione were best correlated with environmental exposure although the correlation of γ-valerolactone and 2,5-dimethylfuran was also statistically significant. The authors considered that the latter two compounds were probably not "true metabolites" but products formed by cyclization of a hydroxylated precursor during gas chromatography. Both 2-hexanol and 2,5-hexanedione might be used as reliable indicators of environmental exposure to *n*-hexane; in practice 2,5-hexanedione, which occurs at higher concentrations, would probably be the most reliable.

The finding that 2,5-hexanedione is the main hexane metabolite in man contrasts with animal studies which suggested that 2-hexanol was the main metabolite (Perebellini, *ibid* 1979, **42,** 349). This difference may indicate a higher susceptibility of man to *n*-hexane neuropathy since 2,5-hexanedione is suspected of being the ultimate neurotoxic metabolite of methyl *n*-butyl ketone and has been found to be selectively retained in the sciatic nerve of rats exposed to *n*-hexane (*Cited in F.C.T.* 1981, **19,** 133).

Mutagenicity of styrene analogues

Norppa, J. (1981). The *in vitro* induction of sister chromatid exchanges and chromosome aberrations in human lymphocytes by styrene derivatives. *Carcinogenesis* **2,** 237.

Styrene, a widely used chemical in the plastics industry, can be metabolized in the body to form styrene oxide, which has been shown to be mutagenic (*Cited in F.C.T.* 1980, **18,** 434). Contradictory evidence has, however, been presented concerning the mutagenicity of styrene itself in several submammalian and mammalian systems (*ibid* 1980, **18,** 434; *ibid* 1981, **19,** 516; Busk, *Mutation Res.* 1979, **67,** 201; Norppa *et al. Carcinogenesis* 1980, **1,** 357). In the present work by the author cited above, three analogues of styrene, *p*-vinyltoluene (VT), 4-methoxy-*trans*-β-chlorostyrene (4-MCS), and *trans*-β-nitrostyrene (NS) were studied for their ability to induce sister chromatid exchange (SCE) and chromosome aberrations in human lymphocytes. VT is used industrially in the production of special coatings, adhesives and latexes and NS is a free-radical scavenger used as a chain stopper in styrene-type polymerization reactions.

Phytohaemagglutinin-stimulated human lymphocyte cultures were established from heparinized whole blood from a healthy male donor. After a 24-hr incubation period, the cultures were treated with the test substances or with acetone as a control, the total treatment time being 24 hr for the aberration studies and 48 hr for the SCE analysis. Bromodeoxyuridine (BrdU) was present in the SCE cultures (so that SCE frequencies could be determined) for the whole 72-hr culture time. A dose-dependent increase in SCEs and chromosome aberrations was observed in cells treated with VT (0·33–4·0 mM) or with 4-MCS (0·05–1·0 mM) but not with NS (0·004–0·044 mM). NS was the most toxic of the analogues tested with no metaphases being observed in cultures treated for 24 hr with 4·0–0·089 mM, whereas the toxicities of VT and 4-MCS were similar to those of styrene and styrene-7,8-oxide respectively.

The author concludes that VT and 4-MCS, but not NS, are mutagenic under the tested *in vitro* conditions. He suggests that, since the mutagen styrene-7,8-oxide can be formed in styrene-treated human lymphocyte cultures (Norppa *et al. loc. cit.*), VT and 4-MCS could be similarly converted *in vitro* to reactive epoxides.

[It would be useful to analyse human lymphocyte cultures treated with VT, 4-MCS or NS to determine whether the respective epoxides are formed *in vitro*.]

Marine pollution and hormonal imbalance

Peakall, D. B., Tremblay, J., Kinter, W. B. & Miller, D. S. (1981). Endocrine dysfunction in seabirds caused by ingested oil. *Envir. Res.* 24, 6.

The apparent inability of seabirds to recognize an oil slick before it has immobilized them results in vast and highly skilled cleaning-up operations, which cannot however overcome the systemic effects that tend to reduce the birds' capacity for survival. Effects on growth and on the liver, kidney, spleen and adrenal glands have been reported, as well as impairment of the osmoregulatory capacity vital to birds feeding in a marine or coastal environment (*Cited in F.C.T.* 1978, **16,** 501; *ibid* 1980, **18,** 106). Thyroid-gland hypertrophy has been noted in birds exposed to some crude oils (Holmes *et al. Envir. Res.* 1978, **17,** 177). and it has been found that while some crude oils and aromatic fractions depress the growth of young birds, other oil samples have no such effect.

The authors cited above stress the importance of studying the disruption of hormonal or neural control mechanisms in addition to the effects of the oils on target organs. Their paper describes the effects of ingestion of various oils or oil fractions on circulating levels of three hormones—corticosterone, thyroxine and adrenocorticotrophic hormone (ACTH)—in nestling herring gulls and black guillemots and in adult Leach's petrels, although the restricted study of the latter made the findings inconclusive.

In laboratory experiments on eight test materials, nestling gulls were given a single intubated dose of a test crude oil (1 ml) or of an aromatic or aliphatic fraction (the amount present in 1 ml of crude oil) in corn oil (total volume 1 ml). Blood samples were taken at intervals for hormone analysis. Some of the oil samples had significant effects on the hormone levels while others had none. The effective samples raised plasma corticosterone levels with a day of dosing and thyroxine levels after 6 days. The former reached a peak (50% above the levels in controls intubated with corn oil) after 4 days and returned to control levels by day 14, at which time the thyroxine levels were still raised. Increases in ACTH levels were also demonstrated. A weathered oil, which increased hormone levels in the laboratory gulls, was used in field studies on the three species, and in spite of the necessarily restricted blood sampling, the results obtained supported the laboratory findings.

All the test materials that increased plasma-hormone levels had been shown in previous studies to reduce growth rates in gulls and guillemots. The other test oils and fractions affected neither hormone levels nor growth. The authors suggest that these findings and the other effects shown to follow ingestion of the 'active' types of oil appear to be consistent with the following sequence of interactions; changes in the intestinal mucosa affect both osmotic balance and nutrient uptake, while direct effects of the oil on the nasal salt gland impose additional stress on the osmoregulatory system; the resulting rise in blood sodium triggers an adrenocortical response and the rise in blood corticosterone leads to proliferation of the nasal salt gland and depresses the growth rate, probably (in view of demonstrated increases in plasma uric acid) by stimulating protein catabolism; salt-gland hypertrophy restores osmotic balance within a few days and corticosterone levels return to normal; the subsequent rise in thyroxine levels is probably a compensatory attempt to increase overall metabolic processes.

Through the skin barrier with NDELA

Bronaugh, R. L., Congdon, E. R. & Scheuplein, R. J. (1981). The effect of cosmetic vehicles on the penetration of *N*-nitrosodiethanolamine through excised human skin. *J. invest. Derm.* **76,** 94.

There have been various reports of contamination of cosmetic products and toilet preparations with *N*-nitrosodiethanolamine (NDELA) at levels ranging up to about 50 ppm (*Cited in F.C.T.* 1977, **15,** 423) and even in some cases up to 130 ppm (*ibid* 1981, **19,** 137). Following earlier studies demonstrating tumour induction in rats and hamsters treated with high total doses of NDELA, a recent study in F344 rats has confirmed the compound's potent carcinogenicity in that species, although it appears the $B6C3F_1$ mice similarly given NDELA in the drinking-water were less susceptible to its carcinogenic activity (*ibid* 1981, **19,** 799).

Since some cosmetics may remain in contact with considerable areas of the body surface for fairly long periods, the carcinogenic potential of NDELA, a non-volatile material, makes its capacity to penetrate the skin barrier a question of major importance. Studies (*Federal Register* 1979, **44,** 21365) have shown NDELA to penetrate excised human skin from an aqueous vehicle and to be aborbed through monkey skin *in vivo*. In an *in vivo* human experiment, another group demonstrated the urinary excretion of NDELA following application of a cream-type facial foundation containing 77 ppm NDELA to the chest and back of a male volunteer (*Cited in F.C.T.* 1981, **19,** 137).

Continuing their *in vitro* studies, the group cited above has now compared the absorption of ^{14}C-labelled NDELA through excised human epidermis following application in three different vehicles—water, propylene glycol and isopropyl myristate—all commonly used in cosmetics. Epidermal samples were removed from human abdominal skin taken at autopsy and the integrity of the epidermal barrier was confirmed by measuring the permeability of the skin to tritiated water. The diffusion cells were designed to reflect *in vivo* conditions as far as possible, and [^{14}C]NDELA was applied to the test membrane in a large excess over that liable to be absorbed, to avoid any significant change in concentration during the exposure.

The permeability constants for NDELA in water and in propylene glycol were both small ($5{\cdot}5 \times 10^{-6}$ and $3{\cdot}2 \times 10^{-6}$ cm/hr respectively) but that for NDELA in isopropyl myristate was some 250 times higher ($1{\cdot}1 \times 10^{-3}$ cm/hr). The increased rate of penetration in the lipoidal vehicle was probably due to a more favourable partitioning into the membrane since the partition coefficients (the ratios of NDELA concentration in the stratum corneum to that in the vehicle) showed a similar pattern—approximately 1·8, 1·0 and 228 for water, propylene glycol and isopropyl myristate respectively.

The authors anticipate that other lipoidal vehicles may also enhance percutaneous absorption of NDELA, and they report a permeability constant of $6{\cdot}2 \times 10^{-5}$ cm/hr for NDELA in a popular lotion (oil-in-water emulsion), a value somewhere between those for the water and the isopropyl myristate vehicles. They point out, however, that some formulation ingredients may affect skin penetration in ways not directly related to their effects on the solubility properties of the vehicle. Using an equation taking into account the concentration of NDELA in a given formulation, the area of application, the skin-contact time and the permeability constant determined for NDELA in that formulation, the amount of NDELA likely to penetrate the skin under the given conditions of use can be estimated. The authors acknowledge that the calculation tends to overestimate the amount absorbed, because it assumes that the surface concentration remains undepleted and disregards any lag time in the penetration rate. However they claim that application of this calculation to the conditions used in the *in vivo* human experiment mentioned above (*ibid* 1981, **19,** 137) using the permeability constant that they had obtained with the popular lotion gave a value for skin absorption of the same order of magnitude as that derived in practice from the urinary excretion of the NDELA.

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LETTER TO THE EDITOR

WORKSHOP ON LABORATORY ANIMAL NUTRITION

Sir,—At the XIIth International Congress of Nutrition held recently in San Diego, California, the International Committee for Laboratory Animal Science sponsored a workshop* on "Nutritional standards for laboratory animal diets". The Committee's object was to encourage discussion of the problems that arise in the feeding of animals on long-term toxicity or carcinogenicity trials, during which minor contaminants or small deviations from optimal nutrient balance can have profound effects on the pathology and lifespan of the test animals. Contributions were made by laboratory-animal users, feed manufacturers and nutritionists from the USA, Japan and Europe.

One major impression to emerge from the discussions was the apparently poor liaison between toxicologists and nutritionists. It was felt that toxicologists are insufficiently aware of the potential influence that a test animal's diet can have on its response to a test compound, since they frequently fail to supply details of diet composition in their publications. Nutritionists, for their part, were considered to have taken too little account of the effects of prolonged feeding of the diets currently formulated for laboratory rodents.

To enable valid comparisons to be made between results of toxicity or oncogenicity trials in different laboratories, it is essential that the composition of the diets used should be precisely known. It is unrealistic, however, to expect a single diet formulation to be suitable for all circumstances. Nutrient requirements of laboratory animals, even rats and mice, are not firmly established and the tendency is to allow generous margins of safety. As a result most laboratory rodents are over-fed, and this, combined with lack of exercise, leads to obesity and encourages the development of 'spontaneous' tumours which can seriously distort the results of long-term toxicity or oncogenicity trials. Convincing evidence was quoted at the workshop, showing that limitation of food intake, or of the protein content of the diet, considerably increases lifespan and reduces tumour incidence. Nevertheless it was pointed out that over-fed under-exercised animals, which are more prone to develop tumours, may be more sensitive indicators of oncogenicity.

There are many commercially available stock diets for laboratory animals but evidence was presented showing great variability in composition between diets from different sources and between different batches of the same diet. In particular, there are large and variable contents of fibre and phytate in stock diets and either of these components may bind toxicants and so reduce their effects. For this reason it was suggested that low-fibre experimental diets may be more suitable for toxicological investigations relevant to man.

On the subject of contaminants it was generally agreed that, since zero levels are impossible to attain, some specifications should be laid down, but they should not be so stringent that available materials fail to conform. If the quality of the raw materials were controlled, occasional analysis of the complete diets might be adequate to check their freedom from undesirable contaminants. Purified ingredients were considered preferable to natural materials, having a lower risk of contamination, but they are more expensive. However, the cost of the diet is relatively small when compared to the overall cost of a toxicity trial, so that increases incurred by the need for analytical controls and the use of purified ingredients were considered comparatively trivial.

As a result of the foregoing discussions the following recommendations were made: (i) closed formula diets (i.e. those for which the composition is not exactly known) should not be used for experimental animals; (ii) editors of toxicological journals should be urged to insist that dietary compositions are recorded in the papers they accept for publication; (iii) attention should be given to the formulation of diets that will support maximal lifespan and minimal tumour incidence; (iv) standards should be set for minimal allowable contents of contaminants in the major ingredients of laboratory-animal diets; (v) research should be encouraged on the topic of drug/nutrient interaction. The effect of test chemicals on nutrient requirements needs investigation, as well as the effect of plane of nutrition on foreign compound metabolism.

M. E. COATES,
(IUNS Representative on ICLAS),
National Institute for Research in Dairying,
Shinfield, Reading, RG2 9AT, England

*A full report of the proceedings will appear in ICLAS Bulletin no. 50, scheduled for publication in March 1982.

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MEETING ANNOUNCEMENTS

SCI FOOD GROUP SYMPOSIUM

A two-day symposium entitled "Food and the Consumer—the Next 50 Years" is being organized by the Food Group of the Society of Chemical Industry as part of their 50th anniversary celebrations. The symposium is to be held at the London International Press Centre on 25 and 26 March 1982. Amongst other topics, the speakers will consider the development of food law, man the consumer, food toxicology and agricultural developments. Further details and application forms may be obtained from The Conference Secretariat, Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS.

COURSE ON AFLATOXIN ANALYSIS AND MYCOTOXINS

This 11-week course at the Tropical Products Institute, London is designed to give scientists from developing countries instruction in the experimental techniques needed for aflatoxin analysis in agricultural products and to provide them with background information on mycotoxins in general. It is intended for those with some experience of chemistry or quality control who are employed by government or commercial organizations or by development institutes which undertake aflatoxin analyses. The course is held once a year and accommodates a maximum of six participants. The next course runs from 29 March 1982 to 11 June 1982. Further details may be obtained from the Training and Visitors Unit, Tropical Products Institute, 127 Clerkenwell Road, London, EC1R 5DB, England.

BRITISH TOXICOLOGY SOCIETY MEETING

The spring 1982 meeting of the British Toxicology Society will be on the subject of "Neurotoxicology". The meeting will be held at the University of Sussex, Brighton on 15–16 April. The two main sessions will be entitled "Methodology in Neurotoxicity" and "Systems/Case Histories". Further details and application forms may be obtained from Dr G. N. Volans, Meetings' Secretary, British Toxicology Society, c/o Poisons Unit, New Cross Hospital, Avonley Road, London SE14 5ER (tel. 01-407 7600).

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FORTHCOMING PAPERS

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

Effects of dietary phospholipids and odd-chain fatty acids on the behaviour maturation of mice. By S. Gozzo, A. Oliverio, S. Salvati, G. Serlupi-Crescenzi, B. Tagliamonte and G. Tomassi.

Differential effects of folic acid on rat kidney water content, protein and microsomal 5′-phosphodiesterase activity. By R. R. Gaddis, R. T. Louis-Ferdinand and F. C. Beuthin.

N-Nitroso-*N*-methyldodecylamine and *N*-nitroso-*N*-methyltetradecylamine in hair-care products. By S. S. Hecht, J. B. Morrison and J. A. Wenninger.

Mutagenicity of commercial hair dyes in *Salmonella typhimurium* TA98. By G. Albano, A. Carere, R. Crebelli and R. Zito.

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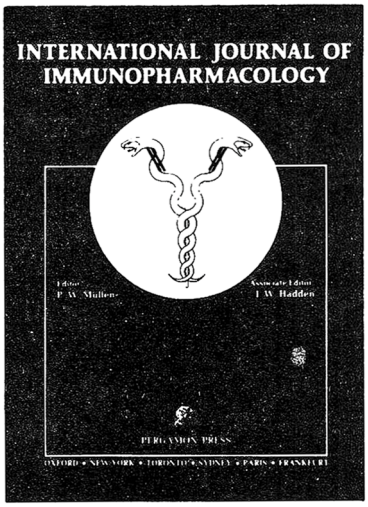

International Journal of Immunopharmacology

Editor: **P W Mullen,** Department of Pharmacology, University of Manchester, England
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This is the official publication of the Canadian Institute of Food Science and Technology. Its aims are as follows:

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Editors: **G A Kerkut**, *Dept of Physiology and Biochemistry, University of Southampton, UK* and **J W Phyllis**, *Dept of Physiology, The University of Saskatchewan, Canada*

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