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RESEARCH SECTION

REVIEW SECTION

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

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Research Section

SACCHARIN: AN EPIGENETIC CARCINOGEN/MUTAGEN?

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(*Received* 18 *November* 1977)

Abstract—Saccharin, together with four of the impurities found in the commercial material and a further nine functional analogues gave negative results in the Salmonella reverse mutation assay of Ames *et al.* (*Mutation Res.* 1975, **31**, 347) and the cell-transformation assay of Styles (*Br. J. Cancer* 1977, **36**, 558). The significance of these and related *in vitro* results is evaluated within the context of the ability of commercial saccharin to cause bladder cancer in rats and dominant lethal effects in mice. In particular, evidence that the carcinogenicity and the dominant lethal effect associated with saccharin may be mediated by an epigenetic mechanism is evaluated, as is the possible role played by impurities in the generation of these and other biological effects. The case for separating carcinogens into two classes, namely those that operate by a *genotypic* mechanism and those that elicit their effects *via* an *epigenetic* mechanism, is discussed. The compounds evaluated in the current study were saccharin base, sodium saccharin, *o*-toluenesulphonamide, *o*-sulphonamidobenzoic acid, 5-chlorosaccharin, 3-iminosaccharin, 6-chlorosaccharin, 5-chlorobenzisothiazoline-1,1-dioxide, 6-aminosaccharin, 6-nitrosaccharin, 3-chloro-ψ-saccharin, 3,6-dichloro-ψ-saccharin, 3-dimethylamino-ψ-saccharin, 3-piperidinyl-ψ-saccharin and 3-pyrrolidino-ψ-saccharin.

INTRODUCTION

In vitro short-term tests for potential carcinogenicity were developed for the evaluation of compounds that have not been submitted to any form of long-term carcinogenicity study. Similarly, inspection of the chemical structure of a compound for electrophilic centres or for structural similarities to known carcinogens is sometimes undertaken to detect possible new carcinogens. In cases where a compound has already been unambiguously established as a carcinogen, the undertaking of these preliminary investigations would be of little value. However, their retrospective use, in particular the use of a mutation-based *in vitro* carcinogenicity assay, may prove helpful in two special situations.

The first is when animal carcinogenicity experiments have given equivocal results, and in such cases data subsequently generated by these methods might influence a reappraisal of the original long-term data. For example, a small increase in the incidence of a spontaneous background tumour would gain added significance either within the context of a positive result for the compound in a Salmonella mutation assay or following the recognition of an alkylating function within its chemical structure. These additional data would confirm that a re-evaluation of the carcinogenicity of the compound should be undertaken. The second situation is when the human significance of a carcinogenic effect that has been demonstrated in animals has been questioned, because of the possibility either that the tumours may have been produced uniquely as the result of administration of the chemical by a biologically inappropriate route or that they are secondary to some specific cytotoxic effect (not necessarily related to gross toxicity) produced by the chemical. These alternative mechanisms would not necessarily require the test compound to be electrophilic in character, to react chemically with DNA or to produce a positive response in *in vitro* mutagenicity assays. Consequently, the absence of these properties from a carcinogen increases the probability of an alternative carcinogenic mechanism.

The literature of carcinogenicity studies carried out on saccharin is extensive and has been reviewed many times (Arnold, Charbonneau, Moody & Munro, 1977; Becker & Thompson, 1975; British Medical Journal, 1975; Hicks, Wakefield & Chowaniec, 1973 & 1975; Munro, Moodie, Krewski & Grice, 1975; Ramsey, 1977; Simpson, 1975; Wright, 1977). Nonetheless, although saccharin is capable of producing tumours under appropriate experimental conditions, there is no general agreement about either the mechanism of action or the significance of these effects to man (Becker & Thompson, 1975; British Medical Journal, 1975; Ramsey, 1977; Simpson, 1975; Wright, 1977). The reluctance shown by many to accept saccharin as a carcinogen of immediate significance to man is based upon three inter-related concerns.

Firstly, the experiments that have most clearly demonstrated its ability to produce tumours have usually been those conducted using either very high dose levels (Munro *et al.* 1975) or experimental techniques that are new and only partially validated (Hicks *et al.* 1973 & 1975). Secondly, although saccharin is capable of inducing mutations in some test

systems (Kramers, 1975), the possibility that these effects may have been produced by one or more of the many impurities known to be present in the commercial material (*Food Chemical News*, 1974; Stavric, Klassen & By, 1976; Subcommittee on Nonnutritive Sweeteners, 1974) has been suggested and in two cases confirmed (Batzinger, Suh-Yun & Bueding, 1977; Kramers, 1975 & 1977). The third concern is that saccharin is chemically nucleophilic in character, and as such gives a predictable negative response in the *Escherichia coli* Pol $A^{+/-}$ test system specifically designed to detect compounds capable of damaging DNA (*Food Chemical News*, 1977; S. Wolff, personal communication, 1977). Further, within the limits of detection, saccharin is excreted unchanged from both man and rats (Ball, Renwick & Williams, 1977). These latter observations mean that the carcinogenic effect observed for saccharin is apparently in total contradiction to the hypothesis that chemically induced cancer is initiated by an electrophilic attack on DNA by the carcinogen or one of its metabolites (Miller & Miller, 1969 & 1971). Such an apparent contradiction cannot be dismissed without also weakening the theoretical foundations upon which all mutation-based *in vitro* carcinogenicity assays rest (Ames, McCann & Yamasaki, 1975; McCann, Choi, Yamasaki & Ames, 1975; Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood, 1976 & 1978).

The above concerns indicate that saccharin may fit into the second category mentioned earlier, in which *in vitro* testing is still justifiable, and it was with this in mind that we undertook an evaluation of saccharin (1), sodium saccharin (2) and thirteen related compounds (nos. 3–15; Fig. 1), using two *in vitro* carcinogenicity tests, namely the Saimonella mutation assay of Ames *et al.* (1975) and the cell-transformation assay of Styles (1977).

EXPERIMENTAL

Chemicals. Whilst some of the compounds used in this study were readily available, others were either difficult to prepare or required critical changes to the

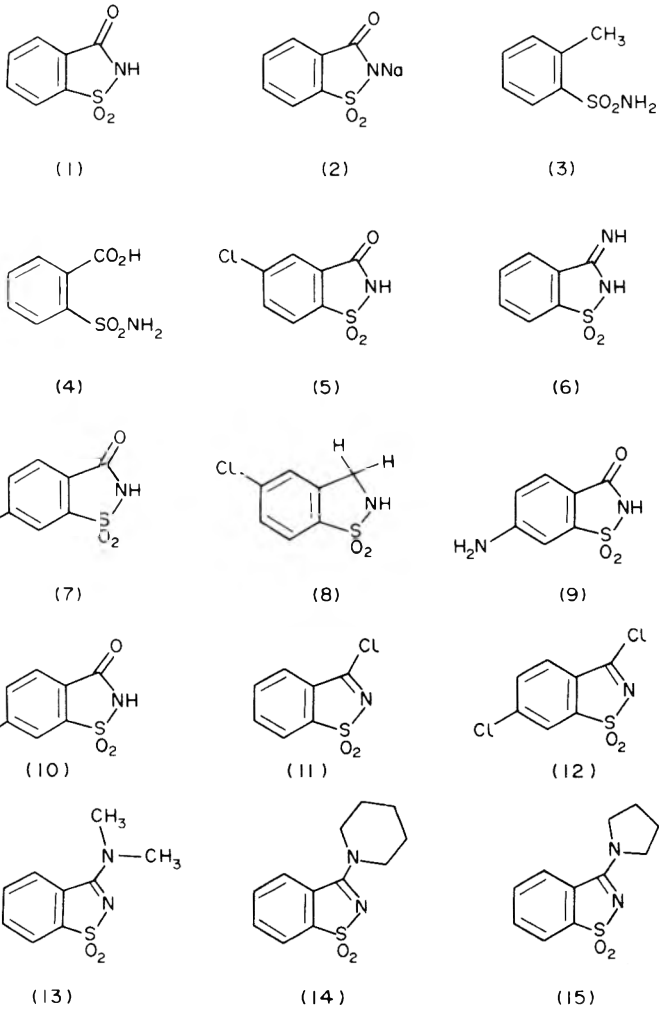

Fig. 1. Structures of saccharin base (1), sodium saccharin (2), *o*-toluenesulphonamide (3), *o*-sulphonamidobenzoic acid (4), 5-chlorosaccharin (5), 3-iminosaccharin (6), 6-chlorosaccharin (7), 5-chlorobenzisothiazoline-1,1-dioxide (8), 6-aminosaccharin (9), 6-nitrosaccharin (10), 3-chloro-ψ-saccharin (11), 3,6-dichloro-ψ-saccharin (12), 3-dimethylamino-ψ-saccharin (13), 3-piperidinyl-ψ-saccharin (14) and 3-pyrrolidino-ψ-saccharin (15).

published methods of preparation. In the latter cases brief details of these changes have been recorded along with evidence to establish the chemical identity of the product. In particular, a possible future source of confusion, the difference in chemical identity between 5- and 6-chlorosaccharin (*Food Chemical News*, 1974), has been resolved.

All nuclear magnetic resonance (NMR) spectra were recorded on a Varian A60, HA 100A or HA 100D or Perkin Elmer R-12 spectrometer, and mass spectral data were obtained using an AEI MS9, MS9025 or Du Pont 21-491B instruments; an m^+ value implies detection of the expected molecular ion of the compound.

Saccharin base (1) was purchased from BDH Chemicals Ltd., Poole, Dorset, and was recrystallized from water and then from acetone without any change in m.p. (232–3°C; cf. Kempf (1908), 228–229°) or thin-layer chromatogram. The major impurity appeared to be compound (4).

Sodium saccharin (2) was obtained by courtesy of ICI Organics Division.

3-Chloro-ψ-saccharin (11) was prepared from saccharin (1) by reaction with thionyl chloride and dimethylformamide in dioxan (The Meiji Confectionery Ltd., 1972). It had m.p. 143° (cf. The Meiji Confectionery Ltd. (1972), 148–149°).

5-Chloro-1,2-benzisothiazoline-1,1-dioxide (8) was obtained by chlorosulphonation of *m*-chlorobenzylamine, followed by cyclization in boiling aqueous ammonia (Nitta, Shindo, Takasu & Isono, 1964a). Recrystallization from ethanol gave colourless prisms (31%), m.p. 151·5–152° (cf. Nitta *et al.* (1964a), 152–152·5°). Required for $C_7H_6ClNO_2S$: C, 41·3; H, 2·9; N, 6·9 and found: C, 41·3; H, 2·9; N, 6·6%. NMR (DMSO d_6) CH_2 (d) 4·42 ppm (2H), aromatic protons + NH (m) 7·5–8·0 ppm (4H) ms, m^+ 203.

5-Chlorosaccharin (5). Alkaline potassium permanganate oxidation of the above thiazoline (8) followed by acidification of the reaction mixture gave the product, which was recrystallized from ethanol to give colourless needles (81%), m.p. 221–222° (cf. Nitta *et al.* (1964a), 213–215°). Required for $C_7H_4ClNO_3S$: C, 38·7; H, 1·8; N, 6·4 and found: C, 38·7; H, 1·9; N, 6·3%. NMR (DMSO d_6) aromatic protons (m) 8·0–8·4 ppm ms, m^+ 217.

6-Chlorosaccharin (7) was prepared by a slight modification of the method of Nitta, Shindo, Takasu & Isono (1964b). As difficulty was experienced when attempting to isolate the intermediate 5-chloro-*o*-toluenesulphonamide, subsequent reactions were conducted using the initially produced mixture of 5- and 6-chlorosulphonamides. This, however, necessitated the careful separation of the required product, 6-chlorosaccharin, from the 4-chloro-3-sulphamoylbenzoic acid formed by oxidation of the contaminating 6-chlorosulphonamide. At pH 3·5, 4-chloro-3-sulphamoylbenzoic acid precipitated slowly, followed upon further acidification by 6-chlorosaccharin. After recrystallization from ethanol, 6-chlorosaccharin (7) was obtained as colourless plates (9% overall yield), m.p. 222–223° (cf. Nitta *et al.* (1964b), 213–215° and De Roode (1891), 218°. Required for $C_7H_4ClNO_3S$: C, 38·7; H, 1·8; N, 6·4 and found: C, 38·6; H, 1·8; N, 6·3%. NMR (DMSO d_6) H^7 (m) 8·55 ppm (1H), H^4, H^5 (m) 8·1 ppm (2H) NH (br) 8·1 ppm (1H) ms, m^+ 217.

The chemical identity of 5-chloro- and 6-chlorosaccharin. The samples of 5-chlorosaccharin and 6-chlorosaccharin used in the present study were unambiguously synthesized and their identity was therefore certain. In the literature the m.p. of both compounds was reported to coincide at 213–215° (Nitta *et al.* 1964a,b), 218° also being reported by De Roode (1891). The present materials were purified so that each melting point was raised, but by an equal amount, to 221–222° and 222–223° respectively. Clearly, melting-point criteria alone cannot be used for the identification of either compound and elemental analysis data will coincide. The C^{13} NMR spectra of each compound are different and can be uniquely associated with each structure, but this technique is still not generally available. We have concluded, therefore, that in the absence of reference samples of each compound the best way to differentiate between these materials is to determine their H^1 NMR spectra in DMSO (d_6), using the multiplet at 8·55 ppm (H-7) to identify 6-chlorosaccharin and its absence to identify 5-chlorosaccharin.

3,6-Dichloro-ψ-saccharin (12). 6-Chlorosaccharin (7) (9·2 g), AR dioxan (40 ml), thionyl chloride (10 ml) and dimethylformamide (0·4 ml) were heated under reflux for 24 hr, and then the solvent was removed *in vacuo* to give the product as a yellow solid (98·7%), m.p. 165–167° raised to 171–172° by recrystallization from benzene. Required for $C_7H_3Cl_2NO_2S$: C, 35·6; H, 1·3; N, 5·9 and found: C, 35·5; H, 1·3; N, 5·9%.

6-Nitrosaccharin (potassium salt) (10), prepared by the permanganate oxidation of 2-methyl-5-nitrobenzenesulphonamide, was obtained by courtesy of ICI Organics Division.

6-Aminosaccharin (9) was purchased from Maybridge Chemical Co., Tintagel, Cornwall.

3-Iminosaccharin (6) was prepared by reaction of 3-chlorobenzisothiazole-1,1-dioxide (11) with ammonia, in a manner similar to that described by Whitehead & Traverso (1960). After recrystallization from water it had m.p. 311–312 (cf. Hettler (1966), 312°); ms m^+ 182.

3-Dimethylamino-ψ-saccharin (13) was prepared by reaction of 3-chlorobenzisothiazole-1,1-dioxide (11) with ethanolic dimethylamine in an excess of boiling ethanol. Upon cooling, the product was filtered and washed with ethanol followed by water (50%), m.p. 294–5° (cf. Pedersen & Lawesson (1974), 297°, and Kruger & Hettler (1969), 301°).

3-Pyrrolidino-ψ-saccharin (15) was prepared by the method of Pedersen & Lawesson (1974) giving 8%, m.p. 270–272 (cf. Pedersen & Lawesson (1974), 268°).

3-Piperidinyl-ψ-saccharin (14). A mixture of saccharin (5 g) and piperidine (5 ml) was heated under reflux in hexamethylphosphoramide (25 ml) in an atmosphere of nitrogen for 7 hr.

After cooling, the resultant solid was collected and the required product was extracted with chloroform (15%), m.p. 202–204° (cf. Hettler (1966), 211°). Required for $C_{12}H_{14}N_2O_2S$. 0·25 H_2O: C, 56·6; H, 5·7; N, 11·0 and found: C, 56·9; H, 5·6; N, 10·9%; ms m^+ 250.

o-*Sulphonamidobenzoic acid* (4) was obtained by hydrolysis of saccharin (20 g) in 4% aqueous sodium hydroxide solution (500 ml) at 100° for 5 hr (Wilson, 1903). Subsequent acidification (with HCl) of the chilled reaction mixture produced the crystalline product, which had m.p. 165–166° (cf. Wilson (1903), 165–7°).

o-*Toluenesulphonamide* (3), m.p. 156° (cf. McKie (1918), 156°), was supplied by courtesy of ICI Organic Division.

Assay methods

Salmonella assay. The method used was that of Ames *et al.* (1975), with minor modifications as described by Purchase *et al.* (1976 & 1978). Arocolor 1254 (Monsanto Chemical Co.) was used for induction of rat-liver enzymes, and 0·15 ml S-9 mix (S-9 fraction; cofactor 27:63) was added per plate. The four strains of *S. typhimurium* used in the present study (TA1535, TA1538, TA98 and TA100) were supplied by Professor B. N. Ames. Test compounds were evaluated using duplicate plates for each of five doses dissolved in 0·1 ml solvent (either dimethylsulphoxide (DMSO) or water, as appropriate) at concentrations of 4, 20, 100, 500 and 2500 μg/plate.

The positive control compounds were 2-nitrofluorene (Sigma Chemical Co. St. Louis, MO) and 2-(1-chloro-2-isopropylaminoethyl)naphthalene (ICI Pharmaceuticals Division). These were tested, using duplicate plates, at concentrations of 20, 100 and 500 μg/plate. Negative controls (DMSO, water and untreated plates) were conducted using triplicate plates. Repeat observations were undertaken for all compounds, generally three times. Background levels of revertant colonies were as reported earlier (Purchase *et al.* 1976 & 1978), and positive control substances gave about 10- to 30-fold increases over these background levels in each case.

Cell-transformation assay. The methods for testing compounds for potential carcinogenicity using the growth of mammalian cells in semi-solid agar have been described (Purchase *et al.* 1976 & 1978; Styles, 1977). The doses used for testing compounds (except the positive control) were 0·025, 0·25, 2·5, 25, 250 and 2500 μg/ml. The positive control was tested to a maximum dose of 250 μg/ml. A positive result is recorded when the transformation frequency/10^6 survivors at the LD_{50} exceeds five times the control frequency/10^6 survivors. The cells used in this study were derived from BHK 21/Cl 13, which had a spontaneous transformation frequency of 50/10^6 survivors. The positive control compound used in all experiments was 2-acetylaminofluorene. In all experiments duplicate plates were used at each dose level.

RESULTS

Salmonella assay

With the exception of the dimethylamino derivative (13), all compounds proved negative in all tester strains each time they were tested. In the case of compound 13 a positive effect was produced in strains TA98 and TA100 the first time they were tested (TA98, maximum count 170 colonies, no dose response, control value 45 colonies, TA100, maximum count 265 colonies, no dose response, control value 60 colonies). These results could not be repeated on subsequent occasions and the related derivatives (14) and (15) were negative in this assay. Saccharin has also been reported to be negative in the related Salmonella strains TA1530 and G46 (Newell & Maxwell, 1972).

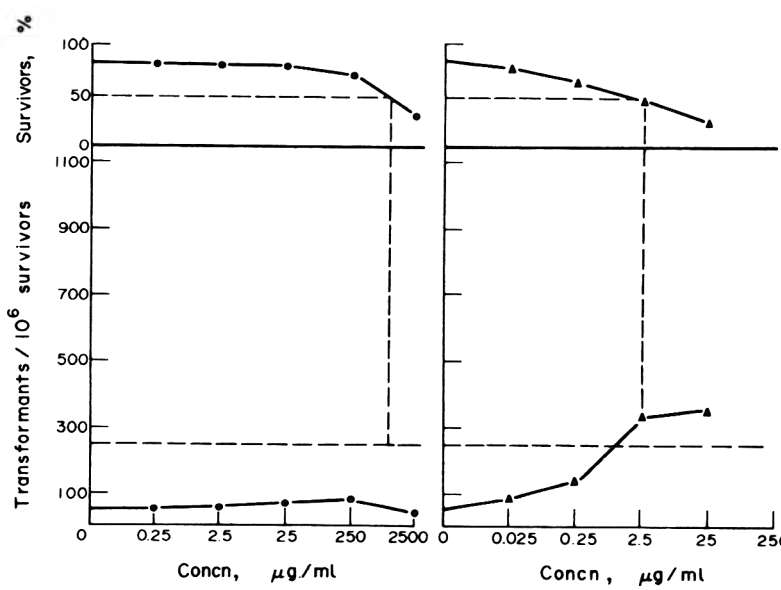

Fig. 2. Survival and transformation dose-response curves of BHK cells treated with (●) 0–2500 μg sodium saccharin/ml and (▲) 0–250 μg 2-acetylaminofluorene/ml (positive control).

Cell-transformation assay

Saccharin base (1), sodium saccharin (2) and all of the derivatives described were non-toxic to the BHK cells in the dose range normally used. The range was extended, therefore, to 2500 μg/ml to obtain the necessary LD_{50} value. All compounds tested (1–13) were negative in the transformation assay and each possessed similar low toxicity. As a typical example, the survival data and transformation frequencies produced by sodium saccharin (2) and those produced by the positive control compound, 2-acetylaminofluorene, are shown in Fig. 2.

DISCUSSION

The continuing debate (Becker & Thompson, 1975; British Medical Journal, 1975; Ramsey, 1977; Simpson, 1975; Wright, 1977) concerning the significance and relevance to man of the bladder tumours that can be produced in rats after the oral administration of relatively high doses of saccharin (Arnold *et al.* 1977; Hicks *et al.* 1973 & 1975; Munro *et al.* 1975) confirms that this chemical has not yet been generally accepted as a conventional organic carcinogen, as has benzo[*a*]pyrene, for example. It is understandable, therefore, that many workers have sought to resolve this problem by studying the effects produced by saccharin in a variety of mutation-based test systems (Kramers, 1975). But these studies themselves have often proved contradictory, probably because not all mutation assays possess equal sensitivity or even necessarily share the same mechanism of action. In addition, some of these investigations have established that impurities present in the commercial material (Batzinger *et al.* 1977; *Food Chemical News*, 1974; Kramers, 1977; Stavric *et al.* 1976; Subcommittee on Nonnutritive Sweeteners, 1974) can play a critical role in determining the response of some *in vitro* test systems to saccharin.

Many impurities have been detected in various samples of commercial saccharin and, although most are present in very low concentrations and none appear to be overtly electrophilic in character, their possible participatory role in the production of some, but probably not all, of the mutagenic effects associated with commercial saccharin is now established (Batzinger *et al.* 1977; Kramers, 1975 & 1977). For example, commercial saccharin is known to produce sex-linked recessive lethal point mutations in Drosophila (Kramers, 1977; Šrám & Weidenhofferová, 1969; Šrám & Zudová, 1972), but these effects have recently been associated with an undefined impurity present in the commercial material rather than with saccharin *per se* (Kramers, 1977). Primarily in response to the positive effects initially observed in Drosphila, Šrám & Zudová (1974) evaluated a commercial sample of saccharin for its ability to cause dominant lethal mutations in mice after ip injection and, in addition, undertook a parallel cytogenetic analysis of mouse spermatogonia.

Both studies produced positive effects but, in contrast to the Drosophila example (Kramers, 1977), it is unlikely that these effects can be dismissed as being due to an impurity. The dominant lethal assay is generally regarded as rather insensitive, optimally producing positive effects for the simple alkylating agents (Bateman & Epstein, 1971; Epstein, Arnold, Andrea, Bass & Bishop, 1972). Therefore, the possibility that a trace impurity could produce effects as marked as those observed by Srám & Zudová (1974) seems unlikely. In fact, the effects observed were unique in being distributed throughout the whole 8 wk of the spermatogenic cycle and are therefore very different from those produced in this assay by a conventional DNA-modifying chemical.

This fact alone suggests that these observations could be open to an alternative explanation. The parallel chromosomal analysis revealed a considerable increase in non-disjunction, an effect that could result from mechanisms other than direct chemical interaction with DNA, and that might have given rise to dominant lethal effects (Bateman & Epstein, 1971). Earlier dominant lethal studies using the oral route of administration, one in mice (Machemer & Lorke, 1973) and another in rats (Newell & Maxwell, 1972), both produced negative results. The negative outcome of these last two experiments may be associated with the use of lower effective doses and different routes of administration (Kramers, 1975). Chromosomal effects other than non-disjunction have been reported for saccharin (Kramers, 1975), but again the possible role of impurities in their generation cannot be assessed retrospectively. The very weak sister chromatid exchange (SCE) effects produced in CHO cells and human lymphocytes by near lethal concentrations of saccharin are not related to the level of impurities in the test material (*Food Chemical News*, 1977; S. Wolff, personal communication 1977). This weak effect therefore represents a property of saccharin *per se*. But this effect may have been triggered by a mechanism other than direct covalent reaction of saccharin with chromosomal DNA. If this were the only trigger for sister chromatid exchange, it would follow that the related, but natural, phenomenon of meiotic crossing-over would have to be associated with the precisely timed intracellular production of one or more DNA reactive species, and this is clearly untenable.

The apparent contradictions that exist in the literature on saccharin mutagenicity may, therefore, be due to the independent or coincident expression of effects associated with saccharin and those associated with one or more electrophilic impurities. Further, those mutagenic effects produced by impurities may not be central to the gross biological effects produced by saccharin. For example, concentration of the lipid-soluble impurities in some particular batches of commercial saccharin led to the isolation of a fraction that gave a weak positive response in the Ames assay (Brusick, 1977; Stoltz, Stavric, Klassen, Bendall & Craig, 1977). However, it has been observed that if this impurity is the agent responsible for the bladder tumours, then it must possess a carcinogenic potency many times greater than that of aflatoxin B_1 (Brusick, 1977); this, although possible, seems unlikely within the context of the chemical structures of the known impurities. Recently, a weak positive response obtained in the Ames assay of some commercial samples of saccharin was lost when a more purified sample was evaluated (Batzinger *et al.* 1977).

The present study has demonstrated that saccharin

base (1) and sodium saccharin (2), together with the ring-opened impurities *o*-toluenesulphonamide (3) and *o*-sulphonamidobenzoic acid (4) and the ring-substituted impurities 5-chlorosaccharin (5) and 3-iminosaccharin (6), are negative in two functionally independent *in vitro* tests for detecting potential carcinogenicity. In addition, as the suspected impurity 5-chlorosaccharin (5) possesses a melting point almost identical to that of 6-chlorosaccharin (7), we evaluated the latter in case the identification of the 5-chloro compound had been based upon melting point criteria alone (*Food Chemical News*, 1974). Benzisothiazoline-1,1-dioxide has also been detected as an impurity in some samples of saccharin (*Food Chemical News*, 1974; Stavric *et al.* 1976), and as this compound was not available for the present study we evaluated its 5-chloroanalogue (8). These two compounds (7 and 8) were also negative in both assays. It has already been demonstrated that some established organic carcinogens are negative in one or other of these two assays (Purchase *et al.* 1976 & 1978); nonetheless, in our experience the failure of both of these tests to detect an established carcinogen is rare, diethylstilboestrol being the only example encountered among the 80 or more carcinogens evaluated in our laboratory to date. (Certain compounds such as dieldrin and phenobarbitone, which produce negative results in these two assays, have been classed as non-carcinogens (Purchase *et al.* 1976 & 1978), a fact which would influence the above statement and which will be discussed later.)

Although the impurities evaluated in this study represent only five of the many compounds that have been detected as contaminants in commercial saccharin, they were chemically representative of the impurities so far defined. Because a crude impurity fraction has been isolated which gave a weak positive response in the Ames assay (Brusick, 1977; Stoltz *et al.* 1977), we synthesized and tested a further five derivatives of saccharin, each bearing potentially or overtly electrophilic substituents. Although these compounds are not likely to be present in the commercial material, each might be expected to impart DNA-modifying properties to the saccharin nucleus. These compounds might therefore be chemically representative of the elusive mutagenic impurity. The additional compounds were 6-aminosaccharin (9), 6-nitrosaccharin (10), 3-chloro-ψ-saccharin (11), 3,6-dichloro-ψ-saccharin (12) and 3-dimethylamino-ψ-saccharin (13).

Despite the fact that carcinogenicity, mutagenicity and cell-transforming ability have been variously associated with these chemical substituents when present on other organic nuclei (McCann *et al.* 1975; Purchase *et al.* 1976 & 1978), no effect was observed for these derivatives in the present study, except for a weak positive effect in the Ames assay for the *N*-dimethylamino compound (13), a response we were unable to reproduce. In order to determine whether the initial positive result was significant, we tested the related compounds 3-piperidino-ψ-saccharin (14) and 3-pyrrolidino-ψ-saccharin (15), each of which might be expected to share any DNA-modifying properties possessed by the *N*-dimethylamino compound (cf. the similarity demonstrated by Druckrey (1975) in the biological responses produced by dimethylnitrosamine, nitrosopiperidine and nitrosopyrrolidine). Neither of these compounds produced a positive response in the Ames assay, which indicates that the original positive response obtained for compound 13 was probably an artefact. This sequence of additional negative results indicates that the mutagenic impurity responsible for the Drosophila effects (Kramers, 1977) and the weak Ames test response (Batzinger *et al.* 1977; Brusick, 1977) has yet to be identified. These results also suggest that before any further studies on saccharin are initiated, international agreement should be sought for an analytical definition of saccharin, and if possible a single batch of material should be made available for use in all future studies, unless such studies are specifically designed to answer questions relating to impurities.

Clearly, since saccharin can produce tumours under certain experimental conditions, the above collection of negative results requires an explanation. Three hypotheses to explain this divergence between *in vitro* and *in vivo* findings are advanced below.

The first of these assumes that saccharin is capable of reacting with cellular macromolecules but that the DNA of these two *in vitro* assays is present in a critically different situation from that encountered by saccharin in the *in vivo* situation, i.e. the general constitution of these tests, on this occasion at least, is inappropriate. This explanation suffers from the objection that saccharin is nucleophilic in character and as such would not be expected to react with DNA; it is in fact negative in two DNA-repair assays (*Food Chemical News*, 1977). Further, the failure of both the Ames assay and the Styles assay to detect a carcinogen (capable of reacting with DNA) is rare, as has been discussed above.

The second possible explanation is that one of the undefined impurities present in commercial saccharin is responsible for the observed carcinogenic effect but that it is unable to produce positive effects in the above *in vitro* assays because of its low concentration in the material tested (20 ppm has been suggested for some commercial samples (*Food Chemical News*, 1977; S. Wolff, personal communication 1977). This explanation cannot be ruled out, although the carcinogenic potency of such a compound would need to be very high (Brusick, 1977, Stoltz *et al.* 1977) and such an explanation would leave saccharin *per se* with some *in vitro* activity. (It is probable that many commonly encountered materials contain trace impurities that would, individually, be capable of producing positive effects in an *in vitro* assay, yet their presence will not always constitute a human hazard, neither will they usually be capable of altering critically the gross biological properties of the compound in question.)

The third possible explanation is that the electrophilic theory of DNA attack (Miller & Miller, 1969 & 1971) linked to the somatic mutation theory of cancer induction may not include all organic carcinogens (Pitot & Heidelberger, 1963; Weisburger, 1973). If this is true, it would be useful to define a mechanism of action that could account for the tumorigenicity of some chemicals but that does not require initial reaction of the chemical with DNA. Such a mechanism might possibly be invoked to account for the tumours produced by compounds

such as saccharin, phenobarbitone, chloroform, carbon tetrachloride, dieldrin, DDT, thioacetamide, thiourea and 3-aminotriazole* and would be consistent with negative results obtained with such compounds in *in vitro* mutagenicity assays: further, the absence of a requirement for them to produce an electrophilic species means that a new approach to chemical structure/biological activity relationships will have to be sought for such compounds. Tumorigenic compounds operating by this mechanism may act in a manner analogous to differentiation, where cells of demonstrably equivalent genetic constitution turn into types that become increasingly diverse. There is evidence (Weiss, 1968) that during this process the cell undergoes irreversible changes induced by specific molecular species and that the inductive exposure need only last for a few hours, after which the affected tissue continues the induced course on its own. Therefore, modification of regulator-gene activity for a short period of time by a chemical could give rise to a variety of phenotypes in the absence of genetic damage, events that Heidelberger and others (Magee, 1977; Pitot & Heidelberger, 1963) have suggested could result ultimately in the production of tumours. Further, as the initiation of these effects may be dependent upon the cell being in a receptive state, such as during regeneration, local toxic effects produced by a high dose of the compound may be required in order to allow the expression of these specific regulator-gene effects (Jull, 1975). In this connexion, it may be relevant that the tumours produced by such compounds usually occur in excretory organs such as the liver, kidney and bladder, where maximum exposure to the chemical would be expected, or in secretory organs such as the thyroid gland.

This mechanism might additionally involve the chemical inhibition of certain DNA-repair enzymes (resulting in the inability of the cell to repair spontaneous mutations of its DNA), interference with cellular hormonal functions (Weisburger, 1973) or disturbances of cellular control mechanisms by the intracellular production of free-radial species derived from the chemical in question. Such species might affect the expression of DNA, yet would not necessarily be required to bind with it chemically or mutate it.

Van Abbé (1976) has suggested that it might be useful to separate carcinogens into two classes, depending upon whether or not they can produce positive effects in bacterial mutagenicity assays. Although the appeal of this approach was weakened at the time by the fact that, in selecting an example of a non-mutagenic carcinogen, the author chose safrole, which produces a positive effect in the Ames test (Green, 1974; Purchase *et al.* 1976 & 1978), the idea was recently expanded by Williams (1977), who tentatively labelled the two classes genotoxic and epigenetic carcinogens. These suggestions coincide with the reasoning that led us to undertake the present study and the concerns raised above about the status of saccharin both as a carcinogen and as a mutagen. The terms *genotypic carcinogen* and *epigenetic carcinogen* would probably be more appropriate. This third explanation therefore casts saccharin as producing bladder tumours in rats *via* an epigenetic mechanism.

The relationship between the two mechanisms postulated above is obscure. If their separate existence is accepted, then the question of whether they act synergistically, independently or exclusively will have to be answered. For example, the carcinogen and Salmonella mutagen, hydrazine, produces changes in several enzyme functions of chick-embryo cells and these lead to an altered cell-differentiation profile (Telang & Mulherkar, 1974), but to associate the carcinogenicity of hydrazine exclusively with either the mutational effects or the cellular-disturbance effect would be premature. In fact, the chemical modification of DNA observed with genotypic carcinogens such as dimethylnitrosamine may be a parallel phenomenon not directly related to the induction of cancer. Whilst this suggestion is extreme, so also may be attempts to modify tests such as the Salmonella mutation assay in order to make them sensitive to chloroform and other compounds (McCann *et al.* 1975). The *in vitro* detection of such compounds might be more profitably pursued in tests that have a non-mutagenic cellular change as their end point, such as the biphenyl-hydroxylation test (McPherson, Bridges & Parke, 1974), the degranulation test of Williams & Rabin (1971) or on occasions a DNA repair assay, or a cell-transformation assay. [3-Aminotriazole, a goitrogen (Tsuda, Hananouchi, Tatematsu, Hirose, Hirao, Takahashi & Ito, 1976) and non-mutagenic carcinogen (McCann *et al.* 1975) is positive in the cell-transformation assay of Styles (1978).]

The confidence that is placed in the idea that carcinogens must react chemically with DNA is due largely to the fact that in the vast majority of cases this is either observed or is at least chemically feasible. However, the large number of carcinogens that apparently confirm this hypothesis may reflect preselection for animal testing on the basis of this very requirement. Therefore, the fact that the number of apparent exceptions, such as saccharin, seems to be few may not be significant. The limited evidence available indicates that most of the possible epigenetic carcinogens selected above are species specific in their effects and produce tumours only after prolonged exposure to the chemical at high dose levels. Further, a well documented collection of compounds that have been tested for carcinogenicity as exhaustively as has saccharin and that are clearly inactive has yet to be established. Only when this has been achieved will it be possible to get general agreement about the significance for man of the positive effects observed for compounds such as DDT. The concept that there may be two separate mechanisms by which chemicals can produce tumours has one very important possible implication, that the normal criteria of carcinogenic hazard developed for DNA-reactive (genotypic) carcinogens such as benzo[*a*]pyrene may not apply to

*Whilst these compounds were selected to represent possible epigenetic carcinogens, it is not intended that the individual suitability of a compound for inclusion should be defended in detail. The points raised in this article are intended as general ones, to be supported, it is hoped, by the specific examples selected and not to be dismissed by the demonstration that one or two of the examples do not produce tumours by an epigenetic mechanism.

compounds producing tumours *via* an epigenetic mechanism. In these cases, normal dose-response relationships may be inappropriate; rather, the defining of a critical toxic-effect level may be necessary, below which exposure to the chemical would not be hazardous.

In conclusion, we suggest that the available evidence indicates that the bladder tumours, the dominant lethal and the SCE effects produced by commercial saccharin are probably due to saccharin *per se* and may arise *via* an epigenetic mechanism. In addition, commercial saccharin probably contains undefined electrophilic impurities capable of producing positive results in some *in vitro* assays but likely to have little or no bearing on the carcinogenic and dominant lethal effects observed *in vivo*. Further, these major biological effects may only be exhibited above a given threshold dose, which may be much higher than the 'safe level' suggested by analogy with carcinogens operating *via* a genotypic mechanism (Cornfield, 1977; Graham, Davis, Hansen & Graham, 1975). These considerations may apply to a very much larger group of biologically related but chemically unrelated epigenetic carcinogens. These conclusions must remain tentative, as they are dependent upon a series of hypotheses, but if proven valid they could have a significant effect on our attitude to the alleged human hazard presented by exposure to some environmental carcinogens.

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Note added in proof

It was established recently that saccharin does not undergo covalent binding to DNA of male rat liver or bladder (Lutz & Schlatter, *Chemico-Biol. Interactions* 1977, **19,** 253). Further, saccharin has been found to be negative in the Salmonella strains TA98 and TA100 even in the presence of norharman, which dramatically increases the mutagenic potency of several carcinogens in the Ames assay (Nagao *et al.* In *Progress in Genetic Toxicology*, edited by D. Scott, B. A. Bridges and F. H. Sobels; p. 259; Elsevier/North Holland Biomedical Press, Amsterdam, 1977).

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THE THERMAL ENERGY ANALYSIS OF SODIUM SACCHARIN

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Abstract—Sodium saccharin has been found to elicit a positive response, equivalent to about 0·0001 mol of an *N*-nitroso compound in thermal energy analysis. All efforts to ascribe this response to an *N*-nitroso impurity have been unsuccessful. It is concluded that the weak TEA response observed is due to the decomposition of sodium saccharin itself.

INTRODUCTION

Few food additives have caused such emotional and scientific upheavals amongst the American public as that currently engendered by saccharin (Arnold, Moodie, Stavric, Stoltz, Grice & Munro, 1977; Pines & Glick, 1977; *Science, New York*, 1977; Society for Occupational and Environmental Health, 1977; *Time Magazine*, 1977). At least two comprehensive surveys of the entire saccharin carcinogenesis problem have appeared within the past few years (Stavric, 1977; Subcommittee on Nonnutritive Sweeteners, 1974). Many people are concerned about the possible impurities present in commercially manufactured sodium saccharin, the most common form of saccharin sold today (Hicks, Wakefield & Chowaniec, 1973). The nature of any impurities present in commercial samples of sodium saccharin depends on the method of synthesis used. Thus, the Monsanto Company at one time manufactured sodium saccharin by the Remsen-Fehlberg process, as did the Diawa Chemical Company in Japan and Farbenfabriken Bayer AG in Germany (*Merck Index*, 1976; Subcommittee on Nonnutritive Sweeteners, 1974). A different process, the Maumee process (*Merck Index*, 1976; Subcommittee on Nonnutritive Sweeteners, 1974) has been used by the Sherwin-Williams Company (Cleveland, OH). Apparently, the only major supplier of sodium saccharin at present in the United States is the Sherwin-Williams Company (*Directory of Chemical Producers*, 1977). It was certain lots of Sherwin-Williams sodium saccharin (lots S1022 and GS-1233) that were used in the recent Canadian cancer study (Stoltz, 1977; D. R. Stoltz, personal communication, 1977). In the Maumee process, certain procedures require the use of sodium nitrite and sulphuric acid in the presence of amines or amine derivatives (Subcommittee on Nonnutritive Sweeteners, 1974) and such conditions can lead to the formation of various *N*-nitroso impurities (Boyer, 1969; Walker, Bogovski & Griciute, 1976). A relatively large number of impurities may be present in the final sodium saccharin produced by the Maumee process, including phthalimide, anthranilic acid, 'disulphide salt', *o*-sulphamoyl-benzoic acid and *o*-sulphobenzoic acid (Subcommittee on Nonnutritive Sweeteners, 1974).

Various groups have studied the nature and amounts of organic impurities present in samples of sodium saccharin and saccharin itself (Arnold *et al.* 1977; Subcommittee on Nonnutritive Sweeteners, 1974). The major impurity present in sodium saccharin produced by the Remsen-Fahlberg process has been found to be *o*-toluenesulphonamide, by both an FDA study and a Wisconsin Alumni Research Foundation analysis (Subcommittee on Nonnutritive Sweeteners, 1974). A much smaller amount of this same impurity was determined in sodium saccharin produced by the Maumee process (Subcommittee on Nonnutritive Sweeteners, 1974). More recently, Arnold *et al.* (1977) have indicated that the Sherwin-Williams sample of sodium saccharin used in the Canadian rat study contained no detectable amounts of *o*-toluenesulphonamide, However, both batches analysed contained 8–12 different impurities. The actual lot used in the Canadian animal study contained 40 ppm of total impurities (Arnold *et al.* 1977; Stavric, 1977). To the best of our knowledge, the major impurities present in the currently available Sherwin-Williams sodium saccharin have not been fully characterized.

In view of the possible formation of *N*-nitroso impurities during the manufacture of sodium saccharin by the Maumee process, we undertook a study to determine the presence of any *N*-nitroso materials in a commercial sample of sodium saccharin (Sherwin-Williams), applying methods and techniques similar to those we have used in the past to determine *N*-nitroso compounds in a wide variety of samples (Fan, Goff, Song, Fine, Arsenault & Biemann, 1977; Fan, Morrison, Rounbehler, Ross, Fine, Miles & Sen,

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1977; Fine, Ross, Rounbehler, Silvergleid & Song, 1977; Fine, Rounbehler, Belcher & Epstein, 1976; Fine, Rounbehler, Pellizzari, Bunch, Berkley, McCrae, Bursey, Sawicki, Krost & DeMarrais, 1976; Fine, Rounbehler, Rounbehler, Silvergeid, Sawicki, Krost & DeMarrais, 1977; Fine, Rounbehler, Sawicki & Krost, 1977; Ross, Morrison, Rounbehler, Fan & Fine, 1977).

EXPERIMENTAL

All high-pressure liquid chromatography (HPLC) solvents were obtained from Burdick & Jackson, Inc. (Muskegon, MI), and had been distilled in glass. Blanks were run using the same solvents as were used for the analyses. Low-temperature slush baths were prepared with technical-grade solvents maintained at their freezing point with liquid nitrogen. The sample of sodium saccharin was obtained from the Sherwin-Williams Company (Cleveland, OH), and was identified as sodium saccharin FCC granular unsized, X17US4584, sample no. 77179C, dated 24 February 1977. The distilled water used for the recrystallization experiment was obtained from Belmont Springs (Belmont, MA).

The HPLC was constructed by combining a high-pressure pumping system (Varian, Palo Alto, CA; Model 8500) with an injector (Rheodyne, Berkeley, CA; Model 7120). Effluent from the HPLC column was first passed through an ultraviolet (UV) absorbance detector (Waters Associates, Milford, MA; Model 440), or a variable wavelength UV–visible HPLC detector (Schoeffel, Westwood, NJ; Model SF770). This was followed by a Thermal Energy Analyzer (Thermo Electron, Waltham, MA; Model 502LC). The HPLC columns used were a 10 μ Porasil (Waters Associates), 30 cm long and 4 mm ID, a 10 μ Bondapak CN column (Waters Associates) of the same dimensions, a 5 μ Polar Bonded Phase slurry-packed column (Macherey-Nagel & Co., Düren, Federal Republic of Germany), and a 10 μm Lichrosorb Si60 slurry-packed column (EM Labs, Inc., Elmsford, NY). The latter two columns were also 30 cm long and 4 mm ID, and were slurry-packed in the laboratory. The solvent systems used for each analysis by HPLC are indicated below. Irradiations were carried out with a GE sunlamp and Pyrex glass tubes.

In an attempt to determine any *N*-nitroso materials in commercial saccharin, the following procedures were carried out: extraction of the material responsive to thermal energy analysis (TEA); concentration of the possible *N*-nitroso impurity; recrystallization of the sodium saccharin; comparison of the UV-response of sodium saccharin with the TEA peak; photolysis combined with TEA of the saccharin. Sublimation and denitrosation of the material were also attempted.

RESULTS AND DISCUSSION

Our initial experiments with the Sherwin-Williams sodium saccharin consisted of attempts to extract into organic solvents TEA-responsive materials. Using chloroform, benzene or acetone extraction followed by direct injections into the TEA, no significant responses were observed, very little of the original sodium saccharin being dissolved. However, sodium saccharin was approximately 15% soluble in methanol at room temperature. When the sodium saccharin was dissolved in distilled water or partially dissolved in absolute methanol, a positive TEA response was observed. Using a known concentration of *N*-nitrosodiethanolamine (NDELA) in distilled water as a calibration standard, it was determined that the aqueous solution of sodium saccharin contained a few ppm of the TEA-responsive substance. However, the peak observed was extremely broad, and not quantitatively reproducible upon successive injections of the same solution. Again, these original injections were obtained on direct injection into the TEA, with no solvent flowing through the furnace of the instrument. On the other hand, NDELA under the same injection conditions gave a very sharp reproducible peak, typical of most volatile *N*-nitroso materials, regardless of the solvent used for the solution being injected. Figure 1 shows the TEA peaks observed for a typical aqueous solution of Sherwin-Williams sodium saccharin, and of the NDELA in the same water.

The very broad ragged shape of the sodium saccharin TEA peak, and its change with successive injections suggested that it might have been the result

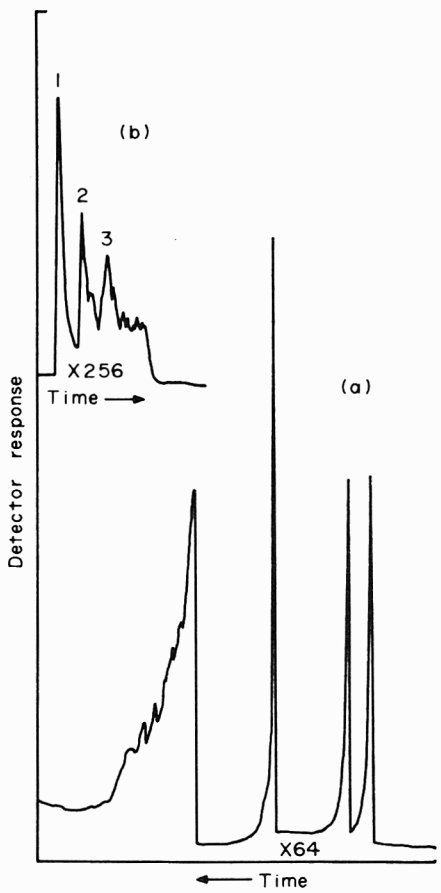

Fig. 1. (a) Direct injection TEA (right to left), two 10 μl samples distilled water, one 10 μl injection (1·6 ppm) NDELA in water, one 10 μl injection Sherwin-Williams sodium saccharin (1 g/ml H_2O). (b) HPLC set up, no column in-line, methanol solvent, 1 ml/min; (left to right) three 10 μl injections sodium saccharin (1 g/ml H_2O).

of slow decomposition of an organic salt in the TEA furnace (Fig. 1). The change in the TEA peaks with subsequent injections suggested that not all of the later injections were undergoing complete thermolysis at the same time. In general, early injections of the water solutions showed greater TEA responses than later injections done in rapid succession. Eventually, after further injections of the sodium saccharin–water solutions, no TEA response could be observed, suggesting that none of the material was being pyrolysed inside the TEA furnace. When the instrument had cooled down sufficiently, it was observed that the injection nozzle and the inside of the pyrolysis furnace were completely coated with tarry pyrolysis products, as well as undecomposed sodium saccharin.

The TEA is designed to operate at temperatures between 400 and 500°C. Such temperatures are needed to vaporize any solvents used for either direct or HPLC injections, as well as for the total thermolysis of any *N*-nitroso bonds in the original sample. However, such relatively low temperatures cannot vaporize organic or inorganic salts completely, though they may cause some rearrangements and decomposition. The use of organic salts for direct injection analysis, or the routine use of aqueous salt solutions as the HPLC eluent, are not compatible with the TEA as currently used.

In all of our initial work, we employed cold traps at −150°C, using the appropriate slush bath, along with a Tenax GC cartridge. These conditions have been shown to remove most of the common thermolysis products that can elicit false-positive TEA responses from organic materials (Bertsch, Chang & Zlatkis, 1974; Fan, Krull, Ross, Wolf & Fine, 1977). Even using such methods, sodium saccharin gave definite, qualitative TEA responses, on direct injection in either aqueous or methanolic solution. However, the shape of the TEA peaks, indicated in Fig. 1, for the sodium saccharin suggested that something other than a simple *N*-nitroso material was at the root of the observed response.

Since the sample was only partially soluble (15%) in absolute methanol, we attempted to concentrate the possible *N*-nitroso impurity in two separate ways. Varying amounts of the salt were extracted with identical volumes of methanol for the same length of time, under identical conditions. In each case, at the end of the extraction procedure, solid sodium saccharin remained undissolved. These solutions were then injected by HPLC–TEA combined with UV–TEA, and the sizes of the TEA peaks were compared with a standard sample of NDELA in methanol injected in the same manner. The HPLC used a 5 μ Polar Bonded Phase (μCN) column (Macherey-Nagel & Co.). An eluent of hexane–methylene chloride–methanol (75:20:5, by vol.) was used in this work. For each of the extracts, the TEA responses were seen to be almost identical, peak height varying between 106–132 ppm. Variations in peak height could be assigned to the absolute amounts injected for each solution, and the way in which the samples were injected.

In a similar experiment, a single 4 g sample of sodium saccharin was extracted three times, with 4 ml of methanol each time. In each extraction, undissolved sodium saccharin remained at the end of the procedure. The three extracts were then analysed as indicated above, and the peak heights were compared with a standard solution of NDELA in methanol. As observed above, the peak heights did not show any pattern suggesting the presence of a methanol-soluble *N*-nitroso impurity.

The above two experiments suggested that the TEA response was not due to an *N*-nitroso impurity, but rather to the thermal decomposition of sodium saccharin itself. Sodium saccharin was then recrystallized three times with distilled water, and each separate recrystallized material was dried under vacuum before analysis. Weighed amounts of each recrystallized material were dissolved in distilled water to yield a ratio of about 1:1 (1 g/1 ml), and these solutions were then injected into the TEA. Because of the large amount of material being injected, and the general problems in using organic salts in the TEA, these injections were made using the HPLC apparatus. However, the HPLC column was removed, and the injections were made with methanol flowing through the remaining arrangement. No significant trends or differences amongst the four samples were observed. These experiments were carried out using two separate TEA instruments, using different pumps and injectors, and the results agreed fully.

If the TEA responses were due to sodium saccharin itself, then the TEA peak should correspond with the UV absorbance of this material. Using the same methanol extracts indicated above, we measured the retention volumes for the TEA and UV peaks. Under all the conditions used, including gradient elution, and with a wide variety of solvent mixtures, the TEA peak always corresponded precisely with the UV peak for the sodium saccharin itself (Fig. 2). The methanol extracts always showed a single UV peak with the μCN columns used, and the complete UV absorbance curve for this peak showed a maximum at 267 μm. This was determined by the use of a variable wavelength UV–visible detector for HPLC. When the original solution in methanol was placed in the sample cell of the instrument, its UV spectrum was identical to that observed from the single peak eluting from the HPLC (Fig. 2). This UV maximum is as reported in the literature for sodium saccharin in 1 N-NaOH solution (*Merck Index*, 1976).

Other HPLC conditions for the analysis of sodium saccharin have been reported in the literature (Brown, 1973; Eng, Calayan & Talmage, 1977; Nelson, 1973; Smyly, Woodward & Conrad, 1976; Twitchett, Gorvin, Moffat, Williams, Sullivan, 1976). Most of these methods used columns of ion-exchange type or reversed-phase type packing materials (Eng *et al.* 1977; Nelson, 1973; Smyly *et al.* 1976). In addition to the use of the μCN packing material, we have also determined HPLC conditions for the analysis of sodium saccharin utilizing a silica-gel-type material. Columns of Lichrosorb Si 60 (10 μ particle size) could be used with a solvent mixture of methylene chloride–acetonitrile–methanol (1:1:1, by vol.), and a solvent flow-rate of 1 ml/min. Under these conditons, sodium saccharin had a retention time of about 8 min, and the TEA peak corresponded identically with the single UV peak for the material.

It is well known that most *N*-nitroso materials are

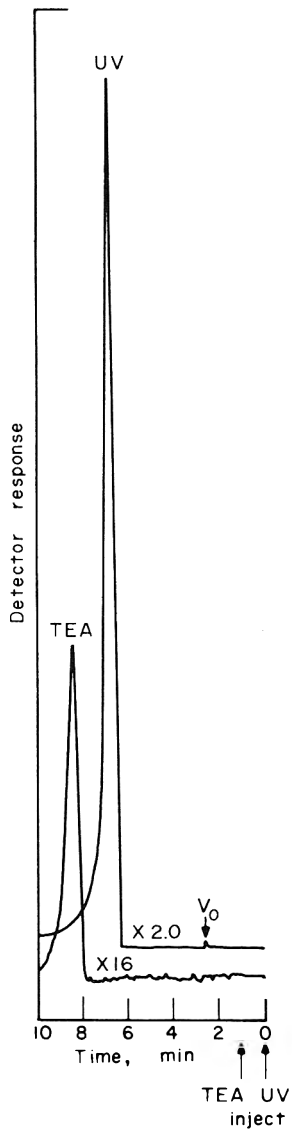

Fig. 2. HPLC chromatogram of Sherwin-Williams sodium saccharin in methanol: 5 μ μCN Polar Bonded Phase/Silica Gel spherical TP (Macherey Nagel & Co.), 1·5 ml/min, hexane–methylene chloride–methanol (68:22:10, by vol.) 2 μl injected.

UV-sensitive, and that under prolonged irradiation conditions, many will undergo rapid photolytic decomposition. Fiddler and his co-workers recently suggest the routine use of photochemistry as an aid in the TEA identification of possible *N*-nitroso materials (Doerr & Fiddler, 1977; Fiddler, Doerr & Piotrowski, 1978). Under the appropriate photolytic conditions, they were able to demonstrate that a combination of photochemistry and TEA analysis can be more useful than the TEA analysis alone. Thus if an unknown impurity is found to exhibit a reproducible TEA response on direct injection or via HPLC (GC), after suitable photolysis of the material in solution, the TEA peak should be reduced in size. Fan & Tannenbaum (1971) described a combination of photolysis and Griess reagent for the confirmation of the nature of *N*-nitroso impurities. We have applied the photolytic experiment to sodium saccharin, and observed a decrease in the TEA response for the material as a solution in water. The experiment was performed at pH 6·7 and 3·0, and in both cases the TEA peak was reduced by approximately 50% in 24 hr. A control solution of NDELA in the same water was irradiated alongside the saccharin samples, and this was also seen to decrease in intensity by about the same proportion. The saccharin and the control solutions were irradiated for identical durations, under identical irradiation conditions and at the same pH values. When the irradiated samples were then analysed by HPLC–UV alongside HPLC–TEA, it was seen that the UV response for the sodium saccharin was decreased by about the same amount as the TEA response, and a new UV-absorbing peak appeared. This again suggested that there was no real *N*-nitroso impurity present in the original sample of sodium saccharin, and that, under photolytic conditions, the saccharin was changed to another material. This newly formed photoproduct apparently does not give any positive TEA response under the conditions employed. Care must therefore be taken when initially interpreting the combined photolysis–TEA experiments as indicated (Doerr & Fiddler, 1977; Fiddler *et al.* 1978).

In an attempt to purify sodium saccharin, and analyse the purified material by TEA, sublimation of the material was pursued. However, because of the expected low vapour pressure of organic salts, it was not possible to obtain any sublimed sodium saccharin.

Downes, Edwards, Elsey & Walters (1976) described a method for the general denitrosation of *N*-nitroso compounds. The method entails treating a solution containing a possible *N*-nitroso material with 3% HBr in glacial acetic acid. This method of denitrosation, when combined with a chemiluminescence analyser such as the TEA, or others, is useful for a very wide range of concentrations of *N*-nitroso derivatives. Johnson & Walters (1971) showed earlier that all the *N*-nitrosamines and *N*-nitrosamides tested responded with the release of NO under the reaction conditions used.

In attempting to apply the denitrosation reaction to sodium saccharin, we noted that the control solution of sodium saccharin in acetone–acetic acid alone, in the absence of added HBr, did not yield a TEA response above that of the solvent mixture by injection through an HPLC injector. That is, the protonated form of sodium saccharin did not produce any detectable TEA signal. Apparently, it is only the sodium saccharin salt that is able to produce a significant TEA peak. At the same time, standard *N*-nitroso compounds, e.g. NDELA, in a variety of organic solvents, water, and mixtures of organic solvents plus acetic acid, always produced a distinct TEA response. These analyses were performed using the HPLC apparatus, in the absence of any column in-line. If there were really an *N*-nitroso impurity present in sodium saccharin, then it should yield the same TEA signal under a wide variety of pH conditions. The formation of the protonated form of sodium saccharin in glacial acetic acid systems should not prevent the TEA response, if it were due to an impurity.

Conclusions

We are aware of a number of materials, other than *N*-nitroso compounds, that will give a TEA response. Stephany & Schuller (1977) have shown that certain *C*-nitroso materials give unusually large TEA responses. Some organic and inorganic nitrates and nitrites also yield non-molar TEA responses (Fine, Rufeh, Lieb & Rounbehler, 1975; Thermo Electron Corporation, 1977). Additional organic chemicals that produce false-positive TEA responses may be found. All of the above results support the conclusion that the sodium salt of saccharin, under the thermolytic conditions within the TEA, produces a small amount of an unknown species which yields a positive TEA response. Very few instruments are totally specific for a single class of organic compounds, and corroborative experiments should be performed in all cases. We have tried to indicate some of the experiments that we believe are readily adapted to direct use with the TEA. Others are undoubtedly available, or will be developed.

Finally, we wish to draw attention to the development of a new HPLC condition for the routine analysis of organic salts. New packing materials, including μCN, μ-diol and μ-NH_2, should prove generally useful for the routine HPLC analysis of polar materials. These columns and solvents do not require the use of aqueous salt solutions, and are thus compatible with instrumentation such as the Thermal Energy Analyzer and the Thermal Nitrogen Analyzer (Fine, 1977).

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EFFET D'UNE CHARGE EN NITRATE SUR LE NITRITE SALIVAIRE ET GASTRIQUE CHEZ L'HOMME

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Résumé—L'évolution des concentrations salivaires et gastriques du nitrate et du nitrite est suivie chez l'homme à la suite de l'ingestion d'un repas test (Lundh, *Acta chir. scand.* 1958, suppl. p. 231) contenant 112 mg de nitrate. Dans le premier groupe (groupe 1) la salive est aspirée afin qu'elle n'atteigne pas l'estomac tandis que dans le second groupe (groupe 2), elle suit son chemin normal. Les échantillons de suc gastrique sont prélevés à l'aide d'une sonde de Salem. Le taux de nitrate gastrique décroit après l'ingestion du repas, mais cette diminution est plus lente que celle du polyéthylène glycol, utilisé comme marqueur de la dilution gastrique. La concentration de nitrite gastrique reste inchangée dans le premier groupe, mais dans le groupe 2 elle augmente rapidement pour atteindre des valeurs individuelles proches de 20 mg/litre 90 minutes après le repas, alors que le pH est voisin de 3. L'apport salivaire de nitrite n'est pas suffisant pour expliquer cette augmentation, qui peut être due à une réduction du nitrate par la flore buccale durant le transit oesophagien. Les résultats sont discutés quant aux risques de formation de nitrosamines *in vivo*.

Abstract—Following ingestion of a test meal (Lundh, *Acta chir. scand.* 1958, suppl. p. 231) containing 112 mg nitrate, the rate of evolution of salivary and gastric nitrite and nitrate was studied in two groups of volunteers. In one group (group 1), the saliva was aspirated to prevent its reaching the stomach, while in the other (group 2) the saliva followed its normal course. Gastric samples were collected through Salem sump tubes. Both groups showed a decrease in the gastric level of nitrate after ingestion of the meal, but the rate of diminution was slower than that of polyethylene glycol, used as a marker of gastric emptying. Gastric concentrations of nitrite did not change in group 1 but in group 2 they increased rapidly, reaching individual levels as high as 20 mg/litre 90 minutes after the meal, when the mean pH was 3. The flow of salivary nitrite was not sufficient to explain this increase, which may have been due to nitrate reduction by the oral microflora during oesophageal transit. The possible relevance of this finding to nitrosamine formation *in vivo* is discussed.

INTRODUCTION

La plupart des toxiques susceptibles d'être rencontrés dans l'alimentation (métaux lourds, pesticides, mycotoxines) sont uniquement d'origine exogène. Un contrôle strict sur la matière et les conditions technologiques de préparation des aliments est la condition nécessaire et suffisante à la diminution de la concentration des polluants dans la ration. Par contre, la présence dans le tractus digestif de composés *N*-nitrosés potentiellement cancérigènes pour l'homme (Magee et Barnes, 1967) peut résulter soit de l'apport alimentaire exogène, soit de la synthèse *in vivo* à partir des amines et du nitrite presents dans la digestion. L'influence du pH acide de l'estomac ainsi que la présence de divers groupements organiques sur la vitesse de réaction et le rendement final ont été clairement établies par de nombreuses études *in vitro* ou *in vivo* chez l'animal de laboratoire (Akin et Wasserman, 1975; Boyland, 1972; Mirvish, 1975).

L'origine des précurseurs aminés est diverse. Ils peuvent être apportés par l'aliment, à la suite d'ajout culinaire (poivre) ou résulter de la dégradation d'acides aminés lors de la cuisson et du stockage. La présence de résidus de pesticides et de fongicides dans les produits de l'agriculture accroît l'apport de groupements nitrosables. De même, l'ingestion de médicamentations à base d'amines secondaires constitue une source supplémentaire de précurseurs qui devient préoccupante lors de traitements chroniques.

La part de nitrite exogène dans l'organisme est minime comparée aux teneurs circulantes résultant de la réduction endogène du nitrate. White (1975) chiffre cet apport quotidien par le bol alimentaire à 4 mg environ et évalue parallèlement la quantité journalière véhiculée par la salive à 8,6 mg dans des conditions d'alimentation normale. Or, le taux de nitrate ingéré peut être élevé (100 mg) puisque les teneurs de certains végétaux atteignent 4 000 ppm (Spiegelhalder, Eisenbrand et Preussmann, 1976).

Harada, Ishiwata, Nakamura, Tanimura et Ishidate (1975) ainsi que Ishiwata, Boriboom, Nakamura, Harada, Tanimura et Ishidate (1975a) démontrent l'influence du taux de nitrate ingéré sur la concentration de nitrate et de nitrite salivaire. Une corrélation positive entre les teneurs de nitrate du repas et le nitrate salivaire ainsi qu'entre ce dernier et le nitrite salivaire est établie par Spiegelhalder *et al.* (1976). Ces résultats suggèrent l'existence d'une relation directe entre le nitrate ingéré et le nitrite recyclé dans la

salive. Ce dernier provient d'une réduction du nitrite soit au niveau intestinal, soit par la flore buccale á partir du nitrate excrété (Tannenbaum, Weisman et Fett, 1976). Walters, Dyke, Saxby et Walker (1976) montrent le rôle majeur de la vidange gastrique dans la disparition du nitrite chez l'homme en suivant la digestion d'un repas contenant 58 ppm de nitrite. La concentration du nitrite au niveau gastrique dépend donc de l'apport salivaire et de la dilution du bol alimentaire.

Afin de préciser l'influence d'un repas contenant une quantité de nitrate compatible avec la pratique alimentaire courante, nous avons suivi les différents éléments impliqués dans une éventuelle réaction de nitrosation des amines: la teneur salivaire et gastrique de nitrite, le pH stomacal et la dilution du repas.

Une étude préliminaire réalisée sur trois sujets seulement nous ayant montré une légère augmentation des teneurs de nitrite gastrique (Klein, Gaconnet, Poullain et Debry, 1976), nous avons étudié deux groupes de sujets, afin de différencier l'influence de l'apport salivaire et l'éventuelle libération ou formation au niveau stomacal (Fritsch, de Saint Blanquat et Derache, 1975). Dans le premier groupe, les volontaires n'avalent pas la salive qui est aspirée. Dans le second groupe, les sujets avalent normalement. Afin de tenir compte des facteurs propres à chaque individu (vidange gastrique), nous avons suivi la dilution du bol alimentaire au moyen d'un marqueur inerte (polyéthylène glycol; PEG).

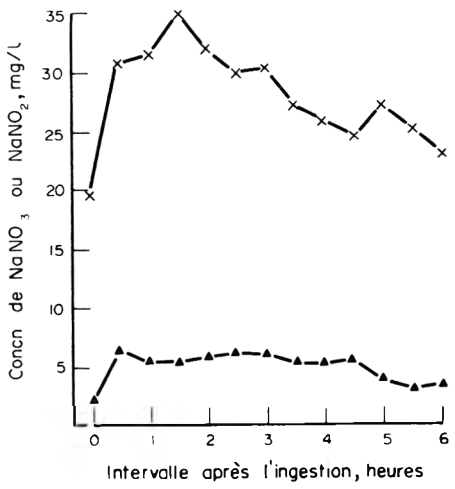

Fig. 1. Evolution des concentrations salivaires de nitrite (▲) et de nitrate (×) à la suite de l'ingestion d'un repas contenant 112 mg de nitrate de sodium.

METHODES EXPERIMENTALES

Sujets. Les concentrations en nitrate et nitrite de la salive sont mesurées sur dix adultes (trois hommes et sept femmes) volontaires ne présentant pas de trouble de la digestion. Sur sept d'entre eux (un homme et six femmes) de moyenne d'âge 25 ans, nous déterminons en outre les teneurs dans le suc gastrique.

Protocole. Une sonde naso-gastrique de Salem est placée chez chacun des volontaires à jeun depuis la veille au soir. L extrêmité de la sonde est repérée par radiographie et positionnée dans la partie basse de l'estomac. Un prélèvement de salive (temps 0) est alors effectué. Les sujets absorbent ensuite 450 ml d'un repas test (Lundh, 1958) contenant pour 100 ml: lait en poudre (10 g), glucose (10 g), huile de maïs (5 g), PEG (2,2 g) et nitrate de sodium (25 mg). Sur cinq sujets (groupe 1), la salive est aspirée à l'aide d'aspirateur à salive et de coton dentaire afin qu'elle ne descende pas dans l'estomac. Les deux sujets restants (groupe 2) avalent normalement la salive. Les prélèvements de salive (6,5 ml) et de suc gastrique (10 ml) sont réalisés une demi-heure après l'ingestion du repas puis toutes les demi-heures.

Analyses. Le nitrite est dosé par le réactif de Griess selon la méthode de Volf, Nogelle et Gautrat (1975). Le prélèvement est dilué à 5 ml puis chauffé à 80°C durant 30 minutes. Les protéines sont précipitées par le mélange ferrocyanure de potassium-acétate de zinc. La coloration est mesurée à 525 nm après addition du réactif de Griess à une partie aliquote du filtrat. Le nitrate est réduit en nitrite par addition de cadmium spongieux à une partie de filtrat tamponné (pH 9,6) et agitation durant 15 minutes. Le cadmium spongieux est obtenu par précipitation à partir de sulfate de cadmium et de lamelles de zinc lavées à l'acide chlorhydrique 0,1 N puis à l'eau distillée. Il est utilisé dans la demi-heure. Le PEG est dosé par opacimètrie après insolubilisation en milieu acide trichloracétique (Hyden, 1955). Les mesures du pH sont réalisées à l'aide de bandelettes pH (Merck AG, Darmstadt).

RESULTATS

La concentration de nitrite du repas varie de 0 à 0,32 ppm selon les préparations.

Les concentrations de nitrate et de nitrite de la salive mesurées à jeun sont respectivement 2,18 ± 1,5 mg/litre et 19 ± 12 mg/litre. Une demi-heure après l'absorption du repas, la teneur de nitrate atteint en moyenne 31 ± 16 mg/litre (Fig. 1). Cet accroissement se poursuit jusqu'à un maximum (35 ± 29 mg/litre) 90 minutes après le début de l'expérience. La concentration du nitrate diminue ensuite lentement mais reste supérieure au niveau de départ, 6 heures après le repas. La teneur en nitrite salivaire croît rapidement dans la première demi-heure puis plus lentement pour atteindre une valeur moyenne maximum de 10 mg/litre (valeurs individuelles extrêmes de 17,8 et 3,9 mg/litre). En fait, ce maximum est atteint à des temps différents selon l'individu (60–180 minutes). La valeur moyenne de 6 mg/litre atteinte à partir de 30 minutes masque des variations individuelles importantes.

Le pH moyen est égal à 5 ± 1 au premier prélèvement (t = 30 minutes; Fig. 2). Il diminue ensuite régulièrement pour atteindre pH 3, 2 heures après le début de l'expérience avec des valeurs individuelles extrêmes de 1,5 et 5. La dilution du bol alimentaire est chiffrée par la diminution de la concentration en PEG du contenu stomacal. Celle-ci décroît régulièrement de 13 ± 6 à 5 ± 4 g/litre (Fig. 2).

Chez tous les individus, la concentration de nitrate gastrique décroît de façon analogue à la teneur en PEG traduisant ainsi la disparition du nitrate par la dilution du repas d'épreuve. Dans l'expression des

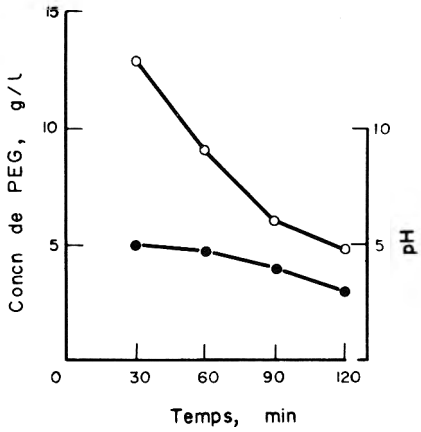

Fig. 2. pH (●) et concentration de polyéthylène glycol (○) dans le suc gastrique.

résultats (Figs 3a,b), nous avons différencié les sujets chez lesquels la salive est aspirée (groupe 1) de ceux qui avalent leur salive (groupe 2). Quatre prélèvements (30, 60, 90 et 120 minutes) ont été réalisés sur les sujets du groupe 1. Sur les sujets du groupe 2 qui présentent une vidange gastrique rapide, nous n'avons pu réaliser que trois prélèvements. Ce phénomène se traduit par la décroissance du PEG dans ce groupe.

Les volontaires qui n'avalent pas la salive ont des teneurs en nitrite très faibles et de même ordre de grandeur que la teneur initiale du repas. Par contre, la concentration en nitrite des sujets que avalent la salive croît rapidement et atteint en moyenne 7 ± 3 mg/litre (valeurs individuelles extrêmes de 5 et 20 mg/litre).

DISCUSSION

Spiegelhalder *et al.* (1976) ont montré récemment l'existence d'une corrélation positive entre les teneurs de nitrate ingéré et celles de nitrite salivaire. Les auteurs estiment que 1 mg de nitrate ingéré provoque par le jeu de recyclages successifs, l'excrétion de 0,2 mg de nitrite et 1,1 mg de nitrate/litre de salive. Cette extrapolation repose sur l'hypothèse d'un flux salivaire constant égal à 50 ml/heure. Or, nous avons constaté chez les sujets en expérience une grande variabilité de ce flux.

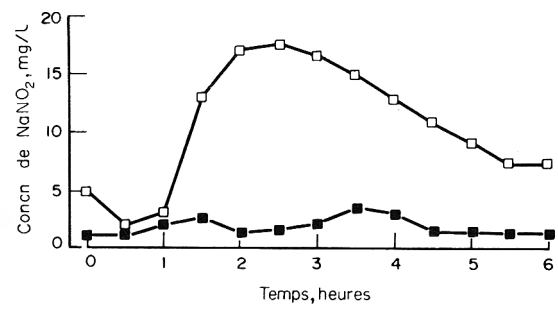

Fig. 4. Evolution des taux de nitrite salivaire chez un sujet salivant peu (□) et chez un sujet ayant un flux salivaire important (■).

Les sujets salivant beaucoup ne montrent pas de variation importante du nitrite salivaire (Fig. 4) alors que les sujets salivant peu présentent des taux de nitrite salivaire élevés. Les concentrations de nitrate sont analogues dans les deux cas. Cette différence, qui rend difficile tout essai de généralisation peut expliquer que nos résultats inférieurs aux publications récentes (Spiegelhalder *et al.* 1976, Tannenbaum *et al.* 1976) soient proches de ceux qui sont obtenus par Walters *et al.* (1976) dans des conditions expérimentales analogues (sujet possédant une sonde).

Influence du repas sur la teneur gastrique du nitrite et nitrate

Pour tenir compte de la dilution gastrique et de la rapidité de la digestion propre à chaque individu, nous avons rapporté les teneurs de nitrite et de nitrate du milieu à la concentration en PEG. Nous constatons alors que le rapport NO_3/PEG est supérieur à tout moment à sa valeur dans le repas et ce aussi bien dans le premier groupe que dans le second (Fig. 5). L'augmentation de ce rapport nitrate/PEG est significative chez les deux groupes mais plus importante chez les sujets avalant leur salive (groupe 2) que chez les membres du groupe 1. Cette différence peut s'expliquer par l'absence de l'apport salivaire de nitrate dans le second groupe.

La concentration de PEG ne diminue que par dilution gastrique et ce composé n'est pas absorbé au niveau de la paroi stomacale. La croissance du rapport nitrate/PEG traduit donc chez les sujets qui n'avalent pas leur salive un apport de nitrate au

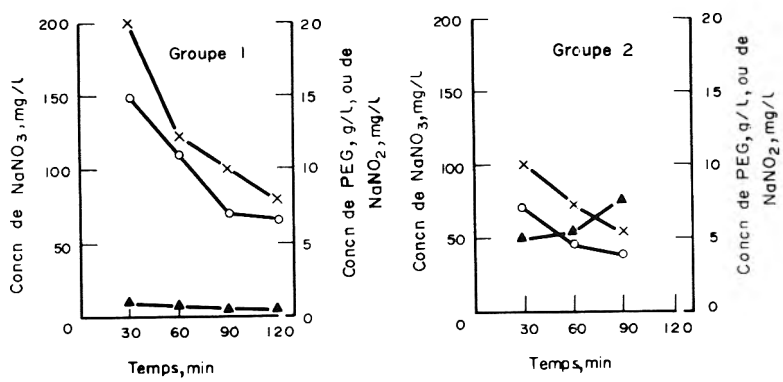

Fig. 3. Evolution des taux gastriques de nitrate (×), nitrite (▲) et PEG (○) au cours de la digestion chez des sujets n'avalant pas leur salive (groupe 1) et chez des sujets l'avalant (groupe 2).

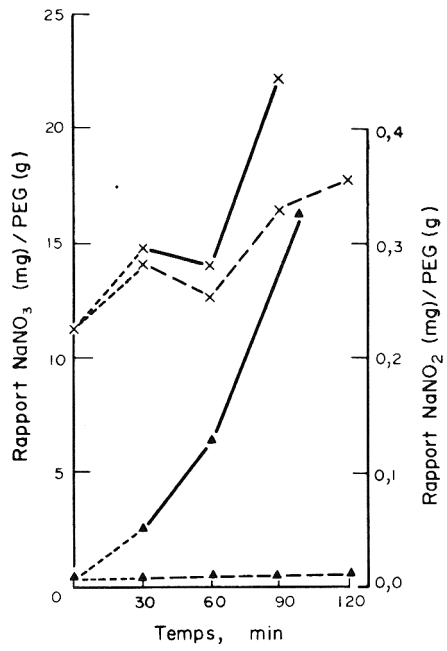

Fig. 5. Rapport des concentrations de nitrate sur PEG (×) et nitrite sur PEG (▲) dans le suc gastrique de sujets (groupe 2) avalant leur salive (——) et de sujets (groupe 1) ne l'avalant pas (– – –). La valeur indiquée à 0 min est la valeur pour le repas.

niveau du milieu gastrique. On peut émettre l'hypothèse d'une sécrétion de nitrate dans le suc gastrique analogue à celle mise en évidence dans la bouche. Chez les sujets avalant leur salive, le flux salivaire complète cet apport gastrique.

En résumé, la concentration en nitrate du contenu gastrique diminue dans le temps mais moins rapidement que le témoin de vidange ce qui conduit à évoquer la possibilité d'une remise en circulation du nitrate par la salive d'une part et les sécrétions stomacales d'autre part.

La concentration de nitrite, et par suite le rapport nitrite/PEG, ne varie pas de manière significative dans le groupe 1. Il n'apparaît donc pas de nitrite au niveau de l'estomac contrairement au nitrate, en dehors de l'apport salivaire mis en évidence chez les sujets du groupe 2 qui avalent leur salive. Cependant, l'apport salivaire moyen de nitrite dans ce groupe n'explique pas les teneurs rencontrées dans l'estomac. En effet, l'apport gastrique en nitrite chez un sujet salivant environ 100 ml/heure serait inférieur à un milligramme dans le même temps. Cette remarque nous conduit donc à admettre une formation durant le transit oesophagien. Cette réduction est réalisée par la flore buccale entraînée lentement par le flux salivaire et dont le rôle dans la conversion du nitrate en nitrite a été montré par Tannenbaum *et al.* (1976). Ces résultats vont à l'encontre de l'hypothèse selon laquelle les concentrations de nitrite salivaire subissent une simple dilution par le suc gastrique, les rendant ainsi inaptes à la réaction de nitrosation (Spiegelhalder *et al.* 1976).

Influence du repas sur le risque de formation endogène de composés N-nitrosés

Ces travaux montrent que le contenu en nitrate du bol alimentaire conditionne l'excrétion salivaire de nitrate et à la suite d'une réduction bactérienne, la teneur de nitrite gastrique. Celle-ci augmente jusqu'à des valeurs dépassant 10 ppm dans un milieu qui évolue dans un sens favorable à la réaction de nitrosation pour les raisons suivantes; par le pH, qui décroit et devient inférieur à 4, et par la diminution de l'effet tampon des composants du bol alimentaire. Nous avons en effet constaté *in vitro* en milieu suc gastrique humain que les constituants du repas avaient un effet inhibiteur sur la réaction de nitrosation. Cet effet diminue lorsque l'on simule la dilution gastrique en augmentant la proportion de suc gastrique dans l'essai (F. Calzarossa, D. Klein, Y. Parmentier et G. Debry, à paraître, 1978).

En fin de repas, les teneurs en amines alimentaires sont sans doute trop faibles pour donner lieu à une synthèse décelable. Les médicaments à base d'amines nitrosables ou leurs métabolites peuvent être réexcrétés par la salive et réagir avec le nitrite conjointement formé durant le transit dans l'oesophage. Le pH neutre de ce milieu est peu favorable mais compensé par la présence de groupements organiques catalyseurs (Archer, Tannenbaum et Wishnok, 1976; Boyland, 1972), ainsi que par la flore entraînée (Ishiwata, Tanimura et Ishidate, 1975b et 1976).

Ce travail confirme au niveau salivaire les études précédentes qui ont montré l'influence des taux de nitrate ingérés sur les concentrations de nitrate et nitrite. Il montre en outre que la teneur de nitrite gastrique semble dépendre essentiellement de l'apport salivaire et est donc liée à la concentration en nitrate du bol alimentaire. Ces résultats relativisent la toxicité du nitrite par rapport à celle du nitrate.

Remerciements—Les auteurs remercient Monsieur le Professeur Bernier et les membres de son équipe de l'Hôpital Saint-Lazare à Paris qui ont réalisé les dosages de PEG. Ce travail a été soutenu financièrement par le Ministère de l'Environnement dans le cadre du contrat no. 7492.

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A SENSITIVE METHOD FOR THE COLORIMETRIC DETERMINATION OF VOLATILE NITROSAMINES IN FOOD PRODUCTS AND AIR

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Abstract—Volatile nitrosodialkylamines were extracted from food using water, and purified and concentrated by distillations from alkaline and acid solution. The nitrosamines were then reduced with Zn and HCl, followed by oxidation to monoalkylhydrazines and coupling of these with *p*-dimethylaminobenzaldehyde. Photometry of the resulting alkylaldazine complex at 458 nm, allows determination of *N*-nitrosodimethylamine in amounts down to 0·1 μg. Factors affecting the yield of the alkylaldazine complex and optimum conditions for the determination are discussed. This method has been used for the determination of *N*-nitrosodimethylamine in food products at concentrations as low as 0·5 μg/kg.

INTRODUCTION

The analysis of various foods and other environmental media for small amounts of volatile nitrosamines has become important since these compounds are recognized as a potential danger to human health. Although gas–liquid chromatography (GLC) is the method generally recommended for trace analytical studies of nitrosamines, positive results based on retention times of chromatographic peaks are of doubtful validity, unless they have been confirmed by other suitable methods, such as mass spectrometry (MS). A valuable improvement in GLC nitrosamine analysis was made by Howard, Fazio & Watts (1970) with the introduction of nitrogen-specific detection, but the determination is still not completely specific. The development by Fine *et al.* (Fine, Rounbehler & Oettinger, 1975; Fine, Rounbehler & Belcher, 1976) of thermal energy analysis (TEA) is an important analytical advance that allows the measurement of small amounts of specific *N*-nitroso compounds in food and air. However, since many laboratories are not equipped with GLC/MS or GLC/TEA, the availability of a sensitive and selective colorimetric procedure remains valuable.

Earlier methods for nitrosamine determination were based on photolytic nitrite release, reduction to amines, reduction to hydrazines and polarographic analysis. Examination of these methods led us to develop further the reduction to dialkylhydrazine by zinc (Fischer, 1879), by combining it with the method for determination of hydrazine described by Watt & Chrisp (1952), which entails coupling with *p*-dimethylaminobenzaldehyde. Oxidation of dialkylhydrazine to monoalkylhydrazine is introduced in an intermediary step. A brief account of our method has already appeared (Ender & Čeh, 1971), and the method has been used for determinations of nitrosamines down to 0·5 μg/kg in Norwegian fish products, cured meats and mushrooms (Ender & Čeh, 1967 & 1968).

EXPERIMENTAL

Materials. Spectrophotometric measurements were made with a Beckman DB spectrophotometer and recorder. Photolysis was carried out with a Hannau Q 600 UV lamp in 1·8 cm diameter test tubes.

The chief reagents used were: phosphotungstic acid reagent (100 g phosphotungstic acid and 50 ml conc. sulphuric acid diluted to 1 litre with distilled water, granulated zinc (Matheson, Coleman & Bell: Reagent A.C.S. CB 825, 20 mesh; suitable grain size is important), Ehrlich's reagent (2 g *p*-dimethylaminobenzaldehyde (E. Merck AG, Darmstadt) dissolved in a mixture made from 10 ml conc. HCl and 90 ml 96% ethanol), activated charcoal granules (1·5 mm granules (Merck 2514; E. Merck AG) ground to smaller particles, but not to powder). Other reagents were of analytical grade.

Procedures

Extraction of nitrosamines from food. A quantity of food containing at least 0·1 μg nitrosodialkylamine, but preferably 1–10 μg, was extracted for 45 min with a volume of freshly distilled water equal to five times the dry weight, at a temperature of about 90–95°C in a boiling water bath. The heated mass was filtered through a Buchner funnel under suction. The filtered mass was then washed twice with hot distilled water. When fat was present, the combined extracts were refrigerated to allow its removal by skimming. Proteins and bases were precipitated using the phosphotungstic acid reagent. The precipitate was filtered off and washed twice with distilled water and the combined extracts* were made alkaline by addition of NaOH pellets to give a concentration of 3 N. Half the volume of the solution was distilled over. NaOH was added to the distillate until it was again 3 N and

*Extracts from single, non-bulky samples are more conveniently prepared by vacuum distillation at 50°C from a slightly alkaline solution. For herring meal containing more than 70 ppm nitrite this procedure should be used.

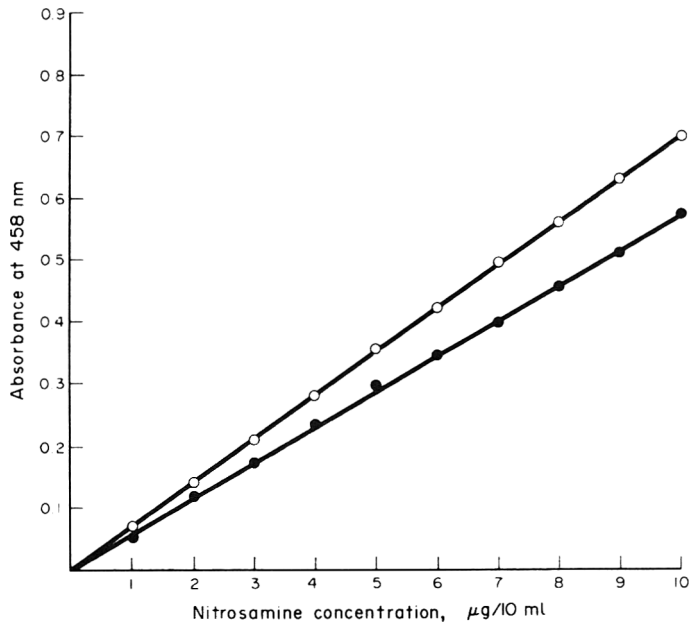

Fig. 1. Calibration curves for NDMA (O) and asymmetric dimethylhydrazine (●) in equivalent amounts.

the process was repeated until a volume of approximately 25 ml was obtained. (When only *N*-nitrosodimethylamine (NDMA) was present, distillation of 10% of the volume from a 10 N-NaOH solution was sufficient for quantitative transfer.) After addition of 2 ml 2 N-H_2SO_4, further distillation was carried out until 2 ml remained. The distillate contained the purified nitrosamines.

For smoked fish and smoked meat products a further purification step was needed to remove indole derivatives. The first distillate was passed through a 30 × 2-cm column containing 4 g activated charcoal. The charcoal was washed twice with distilled water and then transferred to a distilling flask where nitrosamines were distilled quantitatively with 500 ml water at normal pressure. The alkaline and acid distillations were then carried out as described above.

Trapping nitrosamines from air. For analysis of nitrosamines in air, air was forced through cold water to which charcoal had been added, the quantity of air being determined by a flow meter. Adsorbed nitrosamines were then liberated by distillation from the charcoal suspension, as above.

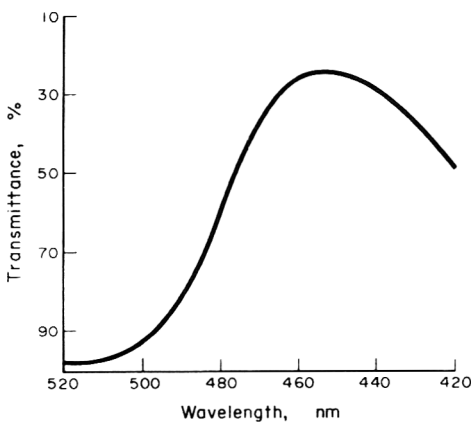

Fig. 2. Absorption spectrum for methylaldazine formed from 10 μg NDMA.

Conversion to alkylaldazine and photometric determination. Granulated zinc (2 g) and sufficient HCl to give a 0·4-N solution were added to 25 ml of the aqueous solution containing nitrosamines in a 50-ml Erlenmeyer flask. The contents were mixed on a laboratory shaker for 1 hr, and after filtration through a glass filter into a 100-ml beaker, the pH was adjusted to 2·0 using NaOH and the volume was increased to 100 ml. To oxidize the dialkylhydrazines, the solution was heated and evaporated at 90–95°C until about 3–5 ml remained. During the last period of evaporation the temperature was lowered by placing an asbestos sheet between the beaker and the hotplate. The contents were cooled to room temperature and 5 ml Ehrlich's reagent was added. After 10 min, water was added to give a total volume of 10 ml and the absorbance was measured at 458 nm.

A calibration curve was obtained by carrying out the above procedure with pure NDMA (Fig. 1). Alternatively, equivalent amounts of dimethylhydrazine may be used, with omission of the reduction step. $ZnCl_2$ must then be added (see below). When assaying a new material, the complete absorption spectrum should be compared with that of the standard, in order to reveal possible interference (Fig. 2).

RESULTS AND DISCUSSION

Oxidation, by atmospheric oxygen, of the dialkylhydrazine to monoalkylhydrazine and an aldehyde is the step most liable to errors. This reaction has been described for dimethylhydrazine by McBride and associates (McBride & Bens, 1959; McBride & Kruse, 1957; Urry, McBride & Kruse, 1957). We have confirmed that formaldehyde and monomethylhydrazine are formed quantitatively from dimethylhydrazine

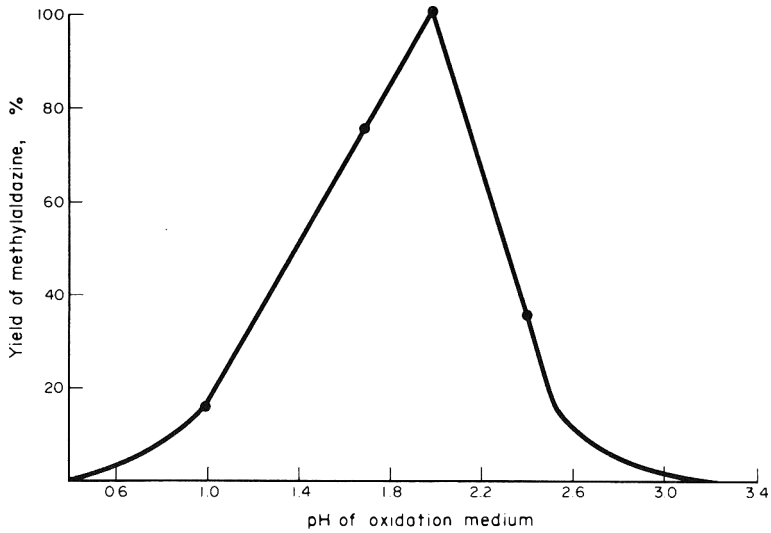

Fig. 3. Effect of pH on oxidation of dimethylhydrazine to monomethylhydrazine.

under our conditions. Formaldehyde was measured with chromotropic acid (MacFadyen, 1945). The oxidation reaction is strongly dependent on pH, as shown in Fig. 3. A yield of aldazine equal to that formed directly from an equivalent amount of monomethylhydrazine was obtained only at pH 2·0. The rate of the oxidation process increases as the temperature increases from 60 to 90°C. However, if the evaporation takes place by boiling, the oxidation is greatly reduced, as would be expected with oxygen dispelled. At 90–95°C, oxidation is related to duration of evaporation and volume reduction, as shown in Table 1. When the starting material is a nitrosodialkylamine, the solution contains $ZnCl_2$ from the reduction step. If oxidation of dialkylhydrazine is attempted in the absence of $ZnCl_2$, no oxidation takes place. Other electrolytes seem to have a similar effect, as $ZnSO_4$ and NaCl could substitute fully. There was no measurable difference in the yield of aldazine with $ZnCl_2$ concentrations between 15 and 100 mM.

The reduction of nitrosamines was conveniently monitored by photometry at the absorption maximum 228 nm. Complete reduction was observed after 30 min of stirring with a magnetic stirrer or 1 hr of shaking on a laboratory shaker. An hour without agitation produced only 66% reduction. Granulated zinc, but not zinc dust, is suitable for the reduction. Reduction to secondary amines does not take place to a significant extent. Assay of secondary amines by the method of Dowden (1938) revealed only trace amounts. This was not changed by varying the HCl concentration in the range 0·1 to 1 N.

Table 1. *The effect of evaporation time at 90–95°C on yield of alkylaldazine*

Evaporation time (hr)	Volume reduction (%)	Alkylaldazine yield (%)
0·5	15	35
1	30	73
1·5	50	98
3·5	99	100

Hydrazine and *p*-dimethylaminobenzaldehyde react in acid alcoholic solution to form the quinoidal cation shown in Fig. 4, in which R is hydrogen (Feigl & Mannheimer, 1953).

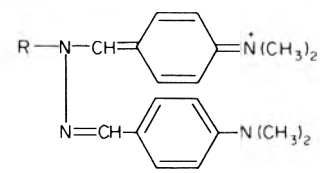

Fig. 4. Aldazine structure.

Compounds with absorption maxima at the same wavelength are formed with monoalkylhydrazines. The structure of these alkylaldazines is probably analogous to that of the unsubstituted aldazine, except that R is now an alkyl group. This has, however, yet to be established.

Volatile aromatic and heterocyclic amines which would react with Ehrlich's reagent are separated from the nitrosamines during distillation at acid pH. Pyrazines, which occur in herring meal and other products exposed to pyrolysis, do not interfere in the present method, although they constitute a problem in GLC determinations of nitrosamines. When pyrrole, indole and their derivatives are present, the charcoal absorption step must be included, as these substances give rise to compounds with absorption at 458 nm.

The dominating nitrosodialkylamine found in food and feed materials is NDMA. When the composition of the nitrosamine fraction is unknown, identification of the components can be carried out according to Neurath & Doerk (1964) by thin-layer chromatography as follows: the purified nitrosamine fraction is photolysed at pH 5 with UV-light to produce amines, which are coupled with 4-nitroazobenzenecarbonic acid-4-chloride. After chromatography, the spots are eluted and photometry is carried out at 336 nm. Higher nitrosoalkylamines were found to be as efficiently reduced by zinc and HCl as was NDMA. The intensity of the colour formed with Ehrlich's reagent was, however, reduced to 95, 65 and 52%, re-

Table 2. *Accuracy of the method for the determination of pure NDMA*

NDMA (μg)	No. of samples	Absorbance (mean ± SEM)	Coefficient of variation
1	6	0·058 ± 0·0047	8·1
4	5	0·221 ± 0·011	5·0
5	10	0·276 ± 0·008	2·9
6	19	0·345 ± 0·0264	7·7
8	19	0·460 ± 0·017	3·7

Table 3. *Comparison of the present hydrazine method with the photolytic nitrite-release method for the determination of NDMA in herring meal*

Sample type*	Method†	No. of samples	NDMA (mg/kg)‡	Coefficient of variation
1	a	10	7·8 ± 0·62	8
	b	4	7·4 ± 0·15	2
2	a	7	1·0 ± 0·14	14
	b	4	0·96 ± 0·06	6
3	a	6	0·09 ± 0·012	13
	b	4	0·15 ± 0·06	40

*Samples of different commercial herring meals weighing, in each case, 25 g.
†Hydrazine method (a) and photolytic nitrite-release method (b).
‡Values are means ± SEM.

spectively, for the diethyl, dibutyl and diamyl compounds.

The smallest amount of NDMA measurable using a 10-mm cuvette was about 0·5 μg. As little as 0·1 μg could be analysed using a 40-mm cuvette. Amounts of NDMA from 1 to 8 μg gave coefficients of variation in the range 2·9–8·1 (Table 2). The data were collected during the course of a year. Recovery tests with added nitrosamines gave values of 90–100% for NDMA in fish meal and about 70% in fish and meat products.

Three different samples of herring meal were analysed by the present method as well as by a modification (Ender & Čeh, 1971) of the photolytic nitrite-release method of Daiber & Preussmann (1964; see Table 3). The methods are seen to agree well at NDMA levels of about 1·0 mg/kg and above, but the photolytic method does not allow determination of NDMA levels below 50 μg/kg, whereas the hydrazine method may be used down to 2 μg/kg for herring meal and 0·5 μg/kg for other materials. NDMA in the range 10–12 μg/kg was found by the hydrazine method in steam-dried commercially-produced herring meal that had not been preserved with $NaNO_2$.

The sensitivity of the method is also demonstrated by its use for the determination of nitrosamines in laboratory and store-room air. The levels found were 1·32 μg/m^3 in the air of the analytical laboratory, 0·38 in the store-room for the samples, 0 in the same store-room, after a thorough airing, and 1·12 in the drying-room for samples.

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EARLY CHANGES OF CARDIAC FUNCTION IN RATS ON A HIGH-FAT DIET

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Abstract—The development of cardiac damage in rats on a high-fat diet was demonstrated by pharmacological methods. Marked changes in the electrocardiogram and decreases in cardiac output and stroke volume developed within 1–2 wk. The increase in cardiac output following injection of epinephrine or norepinephrine was reduced. Various arrhythmias occurred after injection of the catecholamines. Rats fed the high-fat diet for 2 wk and allowed to recover for 2 wk still showed electrocardiographic evidence of residual myocardial damage. It is concluded that cardiovascular function tests are a useful tool for the early demonstration of the cardiotoxicity of a high-fat diet.

INTRODUCTION

In a study of the cardiotoxicity of adriamycin in rats fed a high-fat diet (HFD), significant changes in the electrocardiogram (ECG) were also found in animals that received the HFD without the drug. Marked bradycardia and widening of the QRS complex developed within 1–2 wk. At this time no histopathological changes of the heart muscle were present. On continued feeding of the diet, the ECG changes became more pronounced, and degenerative lesions of the heart muscle developed (Zbinden, Brändle & Pfister, 1977). The present experiment was undertaken to determine the usefulness of cardiovascular function tests for the assessment of early cardiac damage induced by HFD.

EXPERIMENTAL

Experimental procedure. Groups of seven female rats of the ZUR:SIV-Z strain weighing approximately 180 g were kept singly in Macrolone® cages. Three groups were fed HFD, formulated according to Renaud & Allard (1962), and tap-water *ad lib.* The diet contained 53% ground chow (Nafag 890, Gossau), 39·7% butter, 5% cholesterol (USP, Sigma Chemical Co., St. Louis, MO), 2% Na cholate (pract., Fluka AG, Buchs) and 0·3% 6-propyl-2-thiouracil (purum, Fluka AG). Three control groups received ground chow. Cardiovascular function studies of one HFD-fed and one control group were performed after 1 and after 2 wk. The third HFD-fed group was switched to regular chow during wk 3 and 4, and was then subjected to the cardiovascular tests together with the third control group.

Cardiovascular function studies. Rats were anaesthetized by both ip and sc injections of 0·9 g/kg urethane as a 50% (w/v) aqueous solution. Cardiac output was measured using a thermodilution procedure developed by G. Mannesmann (personal communication, 1976) using the HZV Messgerät, Type HZV BN 7206 (Fischer KG, Göttingen, Federal Republic of Germany). For each measurement, 0·1 ml ice-cold 0·9% saline was injected through a polyethylene catheter placed at the entrance to the right atrium through the right jugular vein. A thermistor catheter was introduced through the left carotid artery, the tip being approximately 3 mm distal to the aortic valve. Heparin (Liquemin, Roche) was injected iv in a dose of 10 mg/kg to prevent clotting. The rats were tied to a heated operating table, and rectal temperature was monitored continuously. The trachae was cannulated with a polyethylene catheter. Blood pressure was measured in the right femoral artery with a Statham P 23 Db transducer and recorded with an HE 17 registration unit (F. Hellige GmbH, Freiburg, Federal Republic of Germany). For the ECG recordings, 26-gauge needle electrodes were placed sc over the right scapula and the lumbar vertebrae (lead D; Grauwiler, 1965), using an EK preamplifier and a thermic recorder (F. Hellige GmbH). The paper speed was 10 cm/sec for the baseline ECG and 2·5 cm/sec for the challenge with epinephrine and norepinephrine.

To establish a stable baseline, cardiac output was measured four to six times at 3-min intervals. Body surface was calculated according to Ther (1965). Stroke volume was determined from cardiac output and heart rate.

Five minutes after establishment of stable values, 2 μg/norepinephrine/kg body weight was injected through the venous cathether in 1 sec. ECG and blood-pressure reactions were monitored continuously. Cardiac output was determined 1, 3 and 10 min after the norepinephrine injection. This procedure was repeated after 3–5 min with iv injections of 0·5 μg epinephrine/kg, 10 μg norepinephrine/kg and 1 μg epinephrine/kg. Maximal increases in cardiac output usually occurred after 1 min. Maximal increases in mean blood pressure were expressed as a percentage of the pre-injection pressures.

For the evaluation of the ECG, PR and QT intervals, QRS duration and P, R, S and T wave voltages were measured in five consecutive heart cycles. QT interval was corrected (QT_c) for heart rate using the formula QT_c = QT interval (sec)/$\sqrt{}$ RR interval (sec), of Holzmann (1965). Heart rate was calculated from

RR intervals. Arrhythmias were only evaluated if they occurred three or more seconds after the end of the epinephrine and norepinephrine injections. The nomenclature of the Criteria Committee of the New York Heart Association (1973) was used. R:S wave ratios were expressed as R wave voltage as a percentage of R plus S wave voltages.

At the end of the experiment, rats were killed with an iv injection of 0·3 ml of a saturated KCl solution. Hearts were excised, weighed, fixed in Bouin's solution, embedded in paraffin, sectioned and stained with haematoxylin–eosin.

RESULTS

Rats fed the HFD lost an average of 20 g during wk 1 and maintained their weight thereafter. The group fed regular chow after 2 wk on HFD gained half as much weight as the controls. The only deaths were those of three HFD-fed animals, killed during preparations for the cardiac function studies.

The ECG changes are summarized in Tables 1 and 2. Heart rate decreased significantly after 1 and 2 wk on HFD, but returned to near-normal levels in rats allowed to recover on regular chow. Prolongation of the PR interval was only seen after 2 wk on HFD. The QRS complex was significantly widened after the feeding of HFD for 1 or 2 wk. It did not completely revert to normal values in the group receiving regular chow following 2 wk on HFD. QT was significantly prolonged after 1 and 2 wk on HFD, but returned to normal after the 2-wk recovery period. There were no significant changes of P, R, S and T wave voltages, with the exception of a slight elevation of the T wave in the group receiving 2 wk each of HFD and regular chow.

After norepinephrine administration various arrhythmias were observed (Table 2). In one third of the controls a few ventricular premature contractions followed the administration of 10 μg norepinephrine/kg. There were no such premature contractions in HFD-fed rats. However, ventricular premature contractions were seen in one rat receiving regular chow after 2 wk on HFD.

In the HFD-fed rats various other arrhythmias were observed, most frequently incomplete atrioventricular block (Fig. 1). The same change was also demonstrated in two rats which had recovered for 2 wk on regular chow. Arrhythmias occurred after epinephrine only in the group treated for 2 wk with HFD: one rat showed atrial premature beats, another showed sinus arrest and a third animal had both arrhythmias.

The effects on cardiac output and stroke volume are shown in Table 3. Cardiac output (expressed in ml/min) was significantly reduced in all groups receiving HFD, including that analysed after the 2-wk recovery period. When cardiac output was expressed relative to body-surface area, it was also reduced in all HFD-treated groups, but statistical significance was reached only in the group killed after 1 wk. The same was true for stroke volume, where heart rate is the correcting factor.

Cardiac output increased markedly after iv injection of norepinephrine, and moderately after epinephrine. In HFD-treated rats these increases were moderately reduced. Statistical significance was reached only in one group. In the groups allowed to recover, the increases in cardiac output were the same as or higher than those observed in the corresponding controls.

Blood pressure and its response to norepinephrine were not significantly affected by HFD. The response to epinephrine was more marked in all groups receiving HFD, but this effect of HFD was statistically significant only in the group fed HFD followed by 2 wk on regular chow (Table 4).

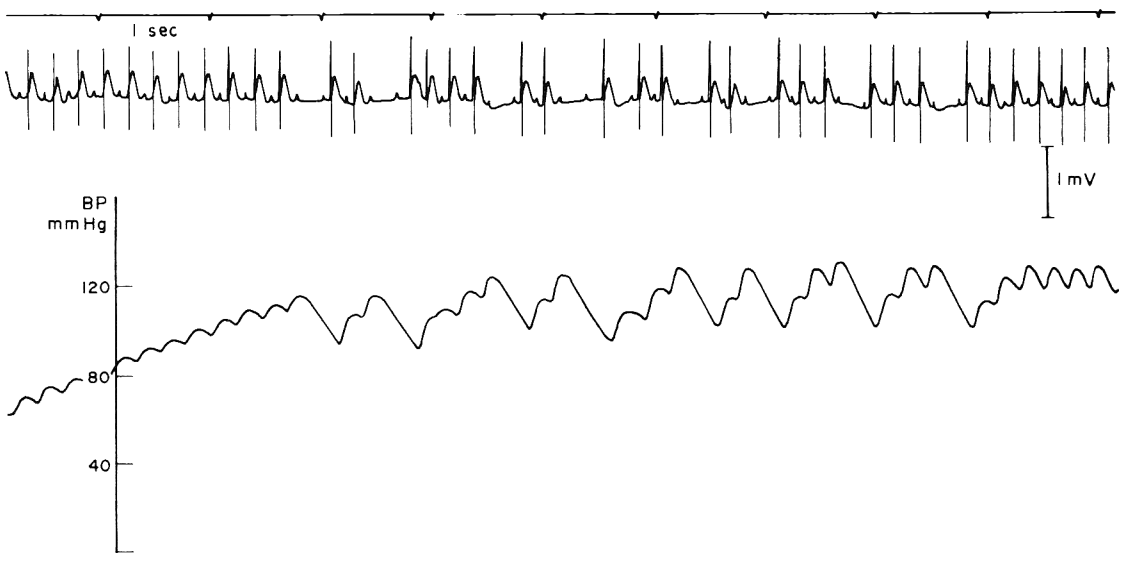

Fig. 1. ECG and blood pressure response after iv injection of 10 μg morepinephrine/kg to a rat fed HFD for 2 wk. The tracing starts 5 sec after the end of the injection. Incomplete atrioventricular block is apparent with a marked decrease in blood pressure each time a ventricular beat is dropped

Table 1. *ECG measurements in rats on HFD and/or regular diet*

Parameter	Diet and duration					
	Control (1 wk)	HFD (1 wk)	Control (2 wk)	HFD (2 wk)	Control (4 wk)	HFD (2 wk) and control (2 wk)
Heart rate† (beats/min)	371 ± 24	306 ± 19***	360 ± 40	261 ± 43***	326 ± 50	295 ± 43
PR (msec)	45·7 ± 18	47·5 ± 36	44·0 ± 2·9	50·6 ± 3·6*	52·2 ± 3·8	50·7 ± 5·4
QRS‡ (msec)	15·9 ± 1·1	18·2 ± 1·5*	15·3 ± 0·8	19·0 ± 1·0***	16·0 ± 1·5	17·9 ± 0·7***
QT_c§ (msec)	184·3 ± 14·8	220·5 ± 29·7**	173·4 ± 15·6	204·4 ± 21·0*	169·6 ± 18·0	162·6 ± 12·1
P (μV)	83 ± 75	127 ± 40	83 ± 53	80 ± 50	66 ± 41	47 ± 57
R (μV)	727 ± 110	605 ± 133	757 ± 96	644 ± 90	699 ± 176	773 ± 101
S (μV)	241 ± 138	345 ± 105	291 ± 47	262 ± 140	233 ± 141	236 ± 123
R (% of R + S)	76 ± 9	63 ± 11	72 ± 5	73 ± 13	75 ± 16	77 ± 11
T (μV)	281 ± 60	213 ± 40	284 ± 66	258 ± 56	236 ± 47	297 ± 42*

†Heart rates are generally lower than in earlier papers (Zbinden *et al.*, 1977) because rats were anaesthetized.
‡Mean QRS durations are approximately 2 msec shorter than those recorded in earlier papers (Zbinden *et al.*, 1977). This is due to the use of a more advanced recorder.
§Actual QT values ranged from 63 to 120 msec. The QT_c values are corrected to eliminate the influence of heart rate. Values are means ± SD for groups of seven rats and those marked with asterisks differ significantly (*U* test of Mann-Whitney) from those of controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 2. *Arrhythmias observed after iv injections of epinephrine or norepinephrine*

Parameter	Diet and duration					
	Control (1 wk)	HFD (1 wk)	Control (2 wk)	HFD (2 wk)	Control (4 wk)	HFD (2 wk) and control (2 wk)
No. of deaths under anaesthetic	0	1	0	2	0	0
No. of animals with arrhythmias* after:						
2 μg norepinephrine/kg	0	0	0	1SA	3VPC	1VPC
0·5 μg epinephrine/kg	0	0	0	0	0	0
10 μg norepinephrine/kg	0	2APB 1AVB	3VPC	1SA 3AVB	4VPC	2AVB
1 μg epinephrine/kg	0	0	0	2SA 2APB	0	0

APB = Atrial premature beats AVB = Incomplete atrioventricular blocks VPC = Ventricular premature contraction SA = Sinus arrest
*From a total of seven in each group.

Table 3. *Cardiac output, stroke volume and heart weight*

Parameter	Diet and duration					
	Control (1 wk)	HFD (1 wk)	Control (2 wk)	HFD (2 wk)	Control (4 wk)	HFD (2 wk) and control (2 wk)
Cardiac output (ml/min)	51·0 ± 13·7	26·1 ± 8·9**	45·3 ± 10·2	30·0 ± 10·0*	50·1 ± 8·3	38·1 ± 8·3*
Cardiac output (ml/100 cm^2)	16·0 ± 4·0	9·7 ± 3·0*	13·5 ± 3·1	10·9 ± 3·1	13·6 ± 2·2	11·6 ± 2·6
Stroke volume (μl)	138 ± 41	86 ± 34*	126 ± 29	119 ± 44	155 ± 28	132 ± 34
Increase in cardiac output (ml/min) after:						
2 μg norepinephrine/kg	8·8 ± 3·7	6·3 ± 3·6	9·9 ± 2·4	7·9 ± 3·6	11·3 ± 3·5	12·7 ± 4·6
0·5 μg epinephrine/kg	5·6 ± 3·7	4·5 ± 4·8	7·7 ± 2·8	3·8 ± 2·2*	10·3 ± 4·3	8·4 ± 1·7
10 μg norepinephrine/kg	16·9 ± 7·7	11·3 ± 4·3	16·3 ± 5·4	17·6 ± 8·9	15·9 ± 4·4	17·9 ± 8·9
1 μg epinephrine/kg	12·5 ± 3·9	5·4 ± 5·6	8·8 ± 5·4	5·7 ± 2·5	7·9 ± 2·5	9·6 ± 6·2
Heart weight (mg)	695·3 ± 38·8	520·4 ± 46·7***	672·7 ± 47·2	511·3 ± 32·9***	788·7 ± 90·6	702·4 ± 76·0

Values are means ± SD for groups of seven rats and those marked with asterisks differ significantly (*U* test of Mann-Whitney) from those of controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. *Blood pressure and responses to epinephrine and norepinephrine*

Parameter	Diet and duration					
	Control (1 wk)	HFD (1 wk)	Control (2 wk)	HFD (2 wk)	Control (4 wk)	HFD (2 wk) and control (2 wk)
Blood pressure (mm Hg)	49·6 ± 3·3	53·3 ± 10·7	53·3 ± 13·3	46·0 ± 12·8	56·1 ± 8·9	58·3 ± 7·3
Percentage increase in mean femoral blood pressure after:						
2 μg norepinephrine/kg	201 ± 24	211 ± 63	195 ± 17	198 ± 45	200 ± 26	197 ± 19
0·5 μg epinephrine/kg	124 ± 13	150 ± 28	123 ± 7	140 ± 29	122 ± 11	129 ± 9
10 μg norepinephrine/kg	274 ± 28	280 ± 23	263 ± 42	303 ± 111	267 ± 40	255 ± 26
1 μg epinephrine/kg	140 ± 18	163 ± 19	135 ± 10	169 ± 42	136 ± 14	158 ± 13*

Values are means ± SD for groups of seven rats and that marked with an asterisk differs significantly (*U* test of Mann-Whitney) from the control: *$P < 0.05$.

Heart weight was significantly reduced in the groups receiving HFD for 1 or 2 wk. After 2-wk recovery on regular chow the heart weight had almost caught up with that of the control (Table 3). In the histological sections of myocardial tissue, coded for blind evaluation, it was not possible to distinguish between the HFD-treated and the control animals.

DISCUSSION

The HFD used in these experiments was originally developed for the study of atherosclerotic and thrombotic lesions in the rat (Renaud & Allard, 1962). Morphological changes, such as large intraventricular thrombi, myocardial infarcts and lipid infiltration in the intima of the aorta occurred after approximately 8 wk. Degeneration of the heart muscle was also observed but treatment had to last more than 30 days before the lesion became visible by routine histopathology (Zbinden *et al.* 1977). The present paper shows that the analysis of cardiovascular functions permits much earlier recognition of the cardiotoxic effects of HFD. It also highlights the complex nature of the cardiac damage. For example, the rapidly developing bradycardia appears to be a pharmacological effect of the propylthiouracil, since it did not occur when the drug was omitted from the diet (Zbinden *et al.* 1977). Other changes such as prolongation of QRS duration and QT_c intervals also occurred on high-fat diets not containing propylthiouracil (G. Zbinden, unpublished data). They are thus probably an expression of metabolic myocardial changes. Of particular interest is the occurrence of various arrhythmias, such as atrial premature beats, sinus arrest and most frequently, incomplete atrioventricular block, following the injection of norephinephrine. These changes never occurred in control animals where norepinephrine only produced ventricular premature contractions. Epinephrine caused arrhythmias less frequently. It is noteworthy that the three rats showing arrhythmias after 1 μg epinephrine/kg belonged to the group receiving HFD for 2 wk. From these observations it is concluded that HFD feeding affected the heart on different functional levels, i.e. the sinus node, the atrioventricular and intraventricular conduction and the repolarization. This multiplicity of effects is indicative of a diffuse myocardial damage.

Feeding HFD also caused a marked reduction in cardiac output and a small decrease in stroke volume, but no change in blood pressure. After the injection of epinephrine or norepinephrine the increases in cardiac output were slightly reduced in the HFD-fed rats, but the pressure responses were not altered. On the contrary, the HFD-fed animals showed a slightly bigger increase in mean arterial pressure after the administration of epinephrine.

It is probable that myocardial atrophy, which developed rapidly in HFD-treated animals, was the reason for the reduced cardiac output and the decreased responses to epinephrine and norepinephrine. This assumption is supported by the results obtained in the group that was permitted to recover for 2 wk. In these rats, heart-weights were only slightly lower than in the controls. Cardiac output and stroke volume improved considerably, and the increases in cardiac output after epinephrine or norepinephrine were normal. However, the evaluation of the ECG provided evidence for residual myocardial damage: the QRS complex was still significantly wider than in controls. In addition, incomplete atrioventricular block developed in two animals after the injection of 10 μg norepinephrine/kg.

The HFD-fed rat is a drastic and complex model for the study of nutritional cardiomyopathy. The diet is toxic, and extreme changes in blood lipids develop rapidly (Zbinden *et al.* 1977). However, the diet rapidly causes predictable cardiac lesions, and is thus a useful system for the development of cardiovascular function tests. With the methods described in this paper it was possible to demonstrate the cardiotoxic effects of HFD at a very early stage. Moreover, residual impairment of cardiac function could be shown in rats that were permitted to recover from the dietary stress. The pharmacological techniques included in this toxicological experiment have thus added valuable information on the cardiotoxic effects of HFD. They may also be useful for the study of other nutritional cardiomyopathies, such as those induced by ethanol or erucic acid.

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GLUCOSE TOLERANCE AND HYPERKINESIS

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Abstract—The medical records of 265 hyperkinetic children carefully selected from out-patients at a treatment centre during the period 1973–1976 were studied for clinical blood chemistry, haematology and results of 5-hr glucose tolerance tests. All the children had been diagnosed as hyperkinetic at least twice including at least one occasion by a physician. Children with other known physical or emotional disturbances were excluded from the study as were adopted children. Of the 16 parameters studied, 13 revealed significant deviations from normal values in very few cases, but haematocrit levels were low in 27% of the cases, eosinophil levels were abnormally high in 86% and in a majority of cases glucose tolerance test results were abnormal, 50% being characterized by low flat curves and 15% by excessive peaks and rapid declines. Some 11% of the remaining abnormal curves were characterized by excessive peaks with slow recovery. Over half of this latter group had elevated cholesterol levels and glucose in the urine. Flat curves with terminal values higher than any others in the curve were seen in about 11% of the subjects. Additional studies should be conducted to investigate the occurrence of abnormal glucose-metabolism patterns as a possible factor in the aetiology of hyperkinesis.

INTRODUCTION

Hyperkinesis has received much attention over the past decade and is being diagnosed with increasing frequency. The term hyperkinesis refers to a broad range of behavioural symptoms seen in children but not found in adults. The syndrome is characterized by inappropriate and excessive physical activity, a short attention span, and an excessive response to environmental stimuli (Holvey, 1972). It has been associated with trauma at birth (Hoffman, 1971; Towbin, 1971), excessive body burdens of lead (David, 1974), emotional stress (Wender, 1971) and diet (Feingold, 1976). In the latter case, hyperkinesis has been claimed to be specifically associated with food colourings and food additives. However, no conclusive evidence exists that bears out a strong cause and effect relationship between the ingestion of food colourings or other additives and hyperkinesis (Kolbye, 1976; Lipton, 1975). A review of the literature did not reveal any studies of hyperkinesis based on the clinical chemistry of those affected with the syndrome. Here we report a clinical study of 265 carefully screened children diagnosed as hyperkinetic.

EXPERIMENTAL

Patients and method. The data used in this study were obtained from the medical records of 265 hyperkinetic children aged 7–9 years, all out-patients at The New York Institute for Child Development between 1973 and 1976. Each child had been diagnosed as hyperkinetic at least once by a parent, teacher or physician. Before being admitted to the Institute for treatment, the diagnosis of hyperkinesis was confirmed by a physician at the Institute. Hyperkinetic children with other known emotional or motor disturbances were excluded from the study. Nine years was used as the upper age limit to eliminate the onset of puberty as another potential variable in the study. Adopted children were not used in the study because of the difficulty of obtaining family histories. No attempt was made to select any particular sex or ethnic ratio among the children studied. The fact that 249 of the children were Caucasian, nine were black and seven were Hispanic is not necessarily an indication that hyperkinesis is more prevalent among whites than among other ethnic groups, but rather reflects the socio-economic level of the children which was predominantly middle stratum.

At the Institute each child was evaluated by a physical therapist, a nutritionist and a physician. The patient's dietary and drug history was recorded as well as his medical history, which included any incidence of colic, allergies, anaemia, gastro-intestinal problems, thyroid dysfunction, diabetes and low blood sugar. The medical history of the patient's family was also recorded as far as possible.

Blood chemistry. Serum samples from the children were analysed for bilirubin, urea nitrogen, calcium, cholesterol, protein-bound iodine, phosphorus, total protein, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and uric acid. Five-hour oral glucose tolerance test (GTT) results were also studied, and urine samples taken at the same intervals as GTT blood samples were analysed for glucose and acetone.

Data analysis. The information on each subject was analysed using a computer to examine distributions, trends and correlations. The normal values for each parameter of clinical chemistry studied were based on those in Todd & Sanford (1969) and the *Merck*

Manual (Holvey, 1972). The widest possible range of normal values was used. In the case of the GTT, median values were used and then a ±20-point spread was added to denote the 'normal range'.

RESULTS

No strong correlations or patterns were evident in family or patient history. Several subjects and their families had histories of allergies, diabetes or anaemia, but the incidence did not differ from that found in a normal population.

A summary of the clinical chemistry data is presented in Table 1. Of the ten parameters of blood chemistry studied, there were no parameters for which significant numbers of subjects (> 20%) had values outside the normal range.

Results of the haematology studies, presented in Table 2, show that in a significant number of cases values were outside the normal range for two parameters investigated: haematocrit and eosinophil counts. The greatest number of cases deviating from the norm occurred in eosinophil counts, with 86% of the subjects exhibiting higher values than normal.

Table 3 shows a summary of the GTT results for blood samples taken at hourly intervals. Lower than normal values predominated, although some individuals showed a greater than normal response. The

Table 1. *Results of serum analyses*

Serum component	Normal range of values	No. of subjects			Subjects with abnormal values (%)
		Tested	Outside normal range		
			Above	Below	
Bilirubin (mg/100 ml)	0·15–1·05	239	6	7	5·4
BUN (mg/100 ml)	6·50–20·0	244	7	1	3·2
Calcium (mg/100 ml)	8·50–11·20	253	3	6	3·5
Cholesterol (mg/100 ml)	120·0–230·0	245	15	4	7·7
PBI (mg/100 ml)	3·50–8·80	95	7	0	7·3
Phosphorus (mg/100 ml)	2·0–7·0	235	0	1	0·4
Protein (total; g/100 ml)	5·90–8·0	251	11	1	4·7
SGOT (mU/ml)	20·0–80·0	240	2	25	11·25
SGPT (IU/ml)	5·0–35·0	73	8	5	17·80
Uric acid (mg/100 ml)	2·0–8·50	247	2	0	0·8

BUN = Blood urea nitrogen PBI = Protein-bound iodine
SGOT = Serum glutamic-oxalacetic transaminase SGPT = Serum glutamic-pyruvic transaminase

Table 2. *Summary of haematology test data*

Blood component	Normal range of values	No. of subjects			Subjects with abnormal values (%)
		Tested	Outside normal range		
			Above	Below	
Haemoglobin (gm/100 ml)	12·0–18·0	261	0	25	9·5
Haematocrit (ml/100 ml)	37·0–52·0	247	0	68	27·5
WBC	5·0–10·0	260	10	34	16·9
RBC	4·2–6·2	207	1	25	12·5
Eosinophils (% of WBC)	0–0·06	245	211	0	86·0

WBC = White blood cell count × 10^3 cells/mm^3 RBC = Red blood cell count × 10^6 cells/mm^3

Table 3. *Summary of glucose tolerance test results*

Time between glucose administration and withdrawal of serum (hr)	Normal range of values	No. of subjects			Subjects with abnormal values (%)
		Tested	Outside normal range		
			Above	Below	
0 (fasting)	65·0–105·0	265	25	6	11·6
0·5	105·0–145·0	194	65	47	57·7
1	140·0–180·0	261	9	221	88·1
2	80·0–120·0	262	37	50	30·2
3	60·0–100·0	258	58	14	27·9
4	70·0–110·0	247	16	29	18·2
5	68·0–108·0	239	16	14	12·5

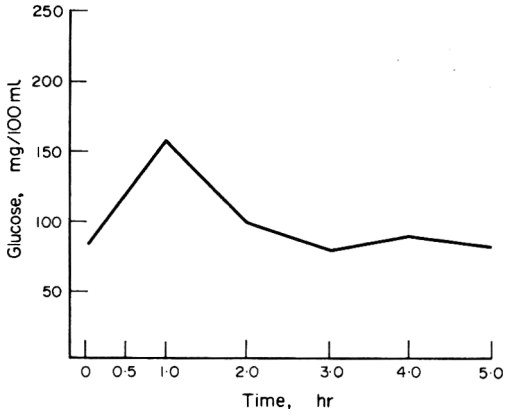

Fig. 1. Normal glucose tolerance test curve.

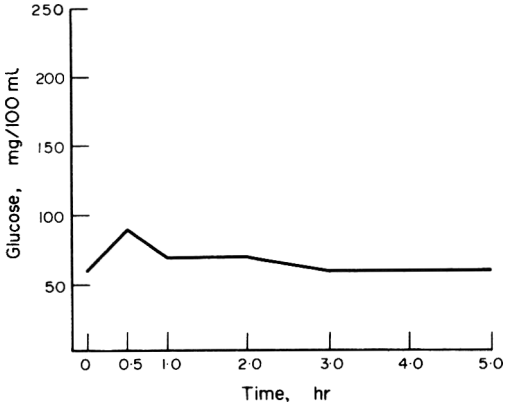

Fig. 2. Glucose tolerance test curve characterized by low flat response.

highest percentage of abnormalities (88%) occurred 1 hr after administration of glucose.

The GTT data were also studied in terms of the response curve as a whole. Data were adequate for this in 261 cases, and in these, 26% of the curves appeared to be normal while the rest showed abnormalities. Figure 1 shows a normal GTT curve. The most frequently observed abnormal curves are illustrated in Figs. 2–5.

The predominant abnormality, accounting for 50% of the abnormal GTT results, was a low flat curve (Fig. 2). Fifteen percent of the curves evidenced

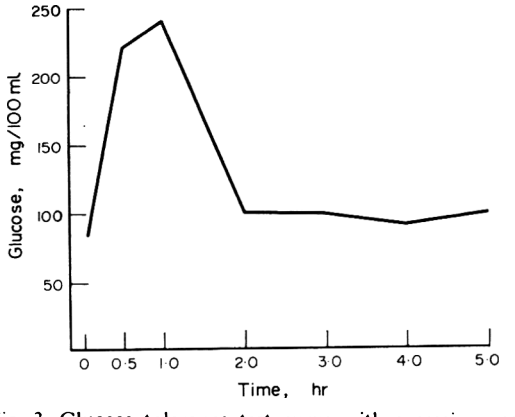

Fig. 3. Glucose tolerance test curve with excessive peak and rapid decline.

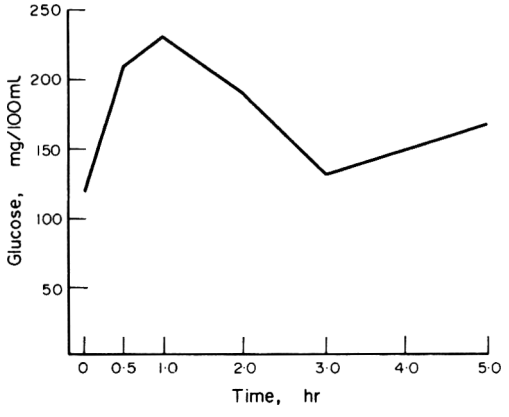

Fig. 4. Glucose tolerance test curve with excessive peak and slow recovery.

abnormally high peaks with extremely rapid-declines (Fig. 3), and almost 11%, or 21 cases, had abnormally high peaks with slow recoveries (Fig. 4). In 15 of the 21 subjects with this latter type of curve, elevated cholesterol levels were also found. Glucose was present in the urine during peak serum glucose levels in 13 of these 21 cases. Almost 11% of the abnormal curves showed a decline immediately after glucose ingestion, with a slow rise in glucose levels and a terminal value higher than fasting values (Fig. 5). Of the remaining 14% of the abnormal curves, 8% were characterized by normal peaks with slow declines and 6% with high late peaks or rapid declines. Acetone was not detected in the urine of any of the subjects studied at any time during GTT.

DISCUSSION

Of the 265 children in the study, 211 were males and 54 were females. The predominance of males is a well-known phenomenon when dealing with hyperkinesis (Kolbye, 1976). It is interesting to note that a 4:1 male to female ratio is consistent with the expected distribution for an X-linked recessive trait (McKusick, 1964), but it should be remembered that an effort was made to screen out subjects with problems other than hyperkinesis.

From an analysis of the clinical data two distinct abnormalities were apparent: raised eosinophil counts and abnormal glucose tolerance test results. Elevated

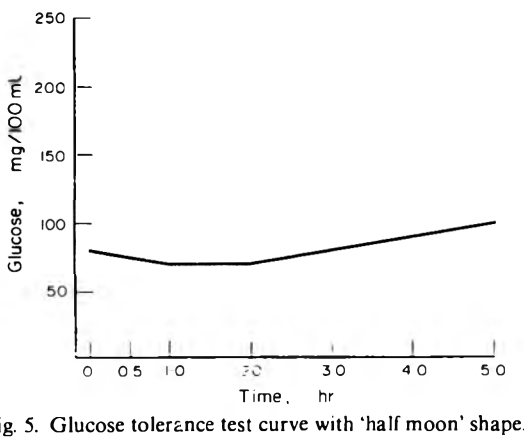

Fig. 5. Glucose tolerance test curve with 'half moon' shape.

eosinophil counts may reflect the incidence of known allergies in the subjects. However, this explanation does not account for elevated levels in all cases, since 45% of those with elevated counts did not report any allergies. Also, since the test subjects were admitted to the Institute and tested throughout the year, high pollen counts during summer months cannot be blamed for the elevated eosinophil levels. Drug therapy can also cause eosinophilia, but only 20% of the children were receiving drug therapy at the time of admission. No explanation of this phenomenon is offered, although it is possible that eosinophil counts could be used as indicators to monitor the progress of a particular treatment for hyperkinesis.

The GTT data clearly indicate that many of the hyperkinetic children tested had abnormal patterns of glucose metabolism, four predominant types of abnormal GTT curves being seen. The low, flat curve shown in Fig. 2 (50% of the abnormal cases) is similar to that seen in hypoglycaemicia. Hypoglycaemia is a potent stimulus for an increased production of epinephrine (Ganong, 1975), and the characteristic nervousness or hyperkinetic behaviour observed in these children is not unlike that seen in hypoglycaemics. It appears that many hyperkinetic children may indeed by undiagnosed hypoglycaemics.

One type of abnormal curve of particular interest is distinguished by a high peak and a slow recovery (11% of the abnormal curves) as shown in Fig. 4. These curves are similar to those found in pre-diabetic children, and in the majority of cases they were associated with glucose in the urine and elevated cholesterol levels, both found commonly in diabetes. The curves with excessively high peaks and rapid declines (shown in Fig. 3) may also be related to early diabetes. However, the relationship here is not as clear as with the curves with abnormally high peaks and slow declines.

It is interesting to note here that many hyperkinetic children have slow growth rates. This has been attributed to Ritalin, the drug of choice for hyperkinetics (Huff, 1975). However, children with diabetes also fail to thrive and the slower growth rates in hyperkinetic children could be related to the possibility, mentioned above, that some of them are early diabetics.

Some of the GTT curves with elevated peaks and slow recovery times could also be due to liver damage from drug therapy; 41% of the subjects had been on drug therapy prior to admission and 20% of the children were receiving drugs at the time of admission. However no strong correlation was found between the distribution of abnormal GTT results and drug therapy.

The type of GTT response that we have named 'half moon' (Fig. 5) may be the result of an overproduction of insulin which could rapidly lower the glucose level in the serum immediately following the ingestion of glucose. This type of response is sometimes observed in certain types of hypoglycaemics.

Hoffman (1971) has associated the hyperkinetic syndrome with trauma at birth, low birth weight, poor nutrition and brain damage. In a number of such subjects, this investigator reported low, flat GTT curves, very much like the ones found in this study.

Crook (1976) has studied food allergy as it relates to hyperkinetic behaviour. He began working with food colourings and additives but later suspected other foods of eliciting hyperkinetic behaviour. He recently reported that sugar was probably the second most frequent dietary cause of hyperkinetic behaviour in susceptible children. Similar findings were reported by Rapp (1976) who found that children challenged with sugar alone showed hyperkinetic responses nearly as great as those in children challenged with sugar and food dye.

On the basis of the GTT results, a diet programme was initiated at the Institute, which now routinely prescribes a diet high in protein and low in carbohydrates, especially sugars. The diet programme appears to have had positive results in reducing and in some cases eliminating hyperkinetic behaviour, and in many cases recurrences of such behaviour have been associated with, among other factors, the ingestion of large amounts of sugar.

The results of this study suggest several models that can be constructed to explain the hyperkinesis phenomenon. The most obvious one involves adrenergic response (Howell, Rever & School, 1972; Schildkraut & Kety, 1976). A diet high in refined carbohydrates stimulates insulin production, which in turn stimulates an adrenergic response. Ultimately the adrenergic response triggers production of epinephrine/norepinephrine. High levels of these hormones can cause the type of behaviour seen in hyperkinetic children.

The authors are aware of the short-comings of the glucose tolerance test. However, even with this reservation, it appears that there are sufficient deviations from generally accepted norms to indicate that glucose metabolism in hyperkinetic children is an area that should be investigated further. There are no doubt many causes of this disorder. This study suggests one possible contributing or causative factor associated with the diet.

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THE SUITABILITY OF SIMULANTS FOR FOODSTUFFS, COSMETICS AND PHARMACEUTICAL PRODUCTS IN MIGRATION STUDIES*

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Abstract—In connection with the control of packaging materials, a radio-tracer technique is being used in migration studies designed to determine the best simulants for the most important foodstuffs and the test conditions of time and temperature most representative of the storage conditions used in practice for any given type of food. Several ^{14}C-labelled plastics additives have been synthesized, incorporated into plastics compounds and compression-moulded into test sheets. These sheets have been stored for various periods in contact with selected food, pharmaceutical, hair-care and cosmetic products, as well as with food simulants proposed by the EEC authorities, at several different temperatures. The amounts of labelled additives migrating from rigid PVC into any of the products were very small. The level of migration from high-density polyethylene was generally much higher, particularly for the relatively small and volatile BHT molecule, but results varied widely with different contact media. In comparative studies with BHT containing HD/PE there was closer agreement of migration levels between the few fat-containing foods and the test fat than with the aqueous food simulants. For most of the pharmaceuticals and cosmetic products appropriate simulants have still to be identified.

INTRODUCTION

In connexion with the control of migration of components from packaging materials into foodstuffs, the EEC authorities have published suggestions about appropriate food simulants and test conditions. Since these are not supported by adequate experimental data, however, they are not always realistic, and the use of unrealistic test methods may have unfortunate consequences. For example, high migration values resulting from the use of too high a test temperature and/or too long a test period could cause the packer of foodstuffs to use unnecessarily expensive packaging materials, while a simulant that is inappropriate for a particular foodstuff could give misleading migration values, resulting in the rejection of a cheap and physiologically unobjectionable packaging material. Therefore, the development of test methods that are easy to use and produce results relevant to practical conditions is in the interests of industry and the consumer, as well as of the legal authorities.

Soon the same problems will arise in the establishment of test conditions for the control of materials used for packaging cosmetics and pharmaceutical products, areas which until recently have received virtually no attention from industry or official bodies and on which no systematic studies have been conducted.

Migration studies have been undertaken, therefore, with the aims of identifying the best simulants for the most important foodstuffs, cosmetic and pharmaceutical products, and of defining the test conditions (temperature and contact time) that best simulate the actual storage conditions used in practice for any given product. These studies involved the use of a radio-tracer technique, which has already been applied successfully to the determination of levels of migration of plastics additives into some complex mixtures. This paper reports some of the results obtained with various types of foodstuffs, pharmaceuticals and cosmetics, and compares these with the migration values obtained in tests involving exposure to the generally approved food simulants under standard conditions.

EXPERIMENTAL

Test materials. Representative plastics additives were synthesized with a ^{14}C label and incorporated into appropriate polymers. The polymers and additives and the composition of the plastics blends are listed in Table 1. For each type of polymer, two blends were produced; these were identical in composition and differed only in the additive that carried the radioactive label. The blends were then pressed into radioactively-labelled test sheets (approximately 350 μm thick) prior to being brought into contact with different foodstuffs, food simulants, cosmetics and pharmaceutical products.

Foods and other products and food simulants. Various types of foods used in these studies are listed in Table 2, together with the storage conditions to which each type is most commonly subjected and the kinds of polymer suitable for its packaging. In the case of fresh cheese, for example, contact between the product and the packaging material occurs on average for 60 days at 10°C, whereas mayonnaise is kept for an average of 120 days and dehydrated soups for as long as 360 days at 20°C. The investigations covered in this interim report relate to cheese, margarine, mayonnaise, whole-milk powder, dehydrated

*Based on a paper presented at the Second International Symposium on Migration held on 3 and 4 November 1976 in Hamburg.

Table 1. *Plastics blends used to produce test sheets containing ^{14}C-labelled additives*

Type of polymer	Plastics blend: Component	Content (% w/w)
HD-PE	Lupolen 5261	99·7
	BHT*	0·2
	Irganox 1076*	0·1
Rigid PVC	Solvic 229	98·0
	Irgastab 17 MOK*	1·0
	Stenol 1618*	0·6
	Loxiol E 10	0·4

HD-PE = High-density polyethylene
Rigid PVC = Unplasticized polyvinylchloride
*Alternative carrier of the ^{14}C label: 2,6-di-*tert*-butyl-4-[^{14}C]methyl-phenol (BHT*), stearyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-[3-^{14}C]propionate (Irganox 1076*), 2-ethylhexyl di-*n*-[1-^{14}C]octyltindithioglycollate (Irgastab 17 MOK*) and [1-^{14}C]stearyl alcohol (Stenol 1618*).

soups and orange juice, as well as to various pharmaceutical tablets, powders, ointments and solutions and foam baths, shampoos and hair-treatment formulations. Comparative studies have involved exposure of the same plastics sheets to the generally accepted food simulants, distilled water, 3% (w/w) acetic acid, 15% (v/v) ethanol, olive oil and the standard triglyceride mixture HB 307 (Figge, 1973). These comparative exposure studies were carried out both under the conditions of time and temperature at which the food in question is stored in practice and under the conditions currently under discussion—0, 5 or 40°C for 10 days, for example.

Test procedures. Details of the test procedures and of the radioanalytical determination of the amounts of migrated additives present in the contact media have already been published (Figge 1976; Figge & Piater, 1971a,b).

RESULTS AND DISCUSSION

Migration into foods and food simulants

Initial trials on sheets of rigid PVC in contact with different foods and the proposed food simulants under conditions relevant to practical storage conditions confirmed that migration of components from rigid PVC to the packaged product is exceptionally low. Thus, after contact periods of 60 or 120 days, less than 0·2% of the stearyl alcohol or Irgastab 17 MOK originally present in the PVC was found in the contact media (Fig. 1). Such amounts of additive are present on the surface of freshly prepared PVC test sheets and can be washed off with 1% aqueous detergent solution. Figure 1 shows that these additives are absorbed to a similar extent by most food products and by the food simulants; any small differences are without toxicological significance in view of the very low levels of transfer involved.

More marked differences are apparent in the transfer of low-molecular-weight components from polyolefins. In initial studies with test sheets made from high-density polyethylene (HD-PE), migration of [^{14}C]BHT during contact for 59 days at 10°C amounted to 9% into fresh cheese and 13 and 15% into the test fats, HB 307 and olive oil, respectively, but only to 0·10 and 0·15% into 3% acetic acid and distilled water, respectively (Fig. 2a). The amounts of BHT transferred from the same HD-PE film into whole-milk powder, dehydrated soups, mayonnaise and the four simulants are shown in Fig. 2b,c.

Comparison of the migration values for the foodstuffs with those for the simulants shows that for studies on HD-PE containing BHT, the aqueous simulants are not suitable substitutes for these food products. Thus, the migration of BHT into the cheese, milk powder, dehydrated soup and mayonnaise was, respectively, 90, 120, at least 160 and at least 220 times higher than that into distilled water.

The amounts of BHT migrating from these sheets into the test fats HB 307 and olive oil are clearly greater by 1·2–1·7 and 1·1–1·6 times, respectively, than those migrating into the foodstuffs under identical storage conditions.

Further investigations on these and related types of food (ice-cream and yoghurt) are planned, using films made of low-density polyethylene, polypropylene, high-impact polystyrene and polyvinylidene chloride.

Table 2. *Usual storage conditions and plastics considered suitable as packaging materials for food products used in these migration studies*

Food product	Storage conditions*: Temperature (°C)	Storage conditions*: Duration (days)	Plastics suitable for packaging: LD-PE	HD-PE	PP	HI-PS	Rigid PVC	Rigid PVDC
Cheese								
Fresh (averaging 50% fat)	10	60		+			+	
Processed (averaging 50% fat)	20	60			+		+	
Mayonnaise (30 or 80% fat)	20	120		+		+	+	
Margarine	15	60			+		+	+
Whole-milk powder	15	120		+				
Dehydrated soup, clear or thickened	20	360	+	+		+	+	
Orange juice	20	120					+	+

LD-PE and HD-PE = Low- and high-density polyethylene PP = Polypropylene HI-PS = High-impact polystyrene
PVC = Polyvinyl chloride PVDC = Polyvinylidene chloride
*Based on the usual storage conditions in factories, warehouses, shops and households.

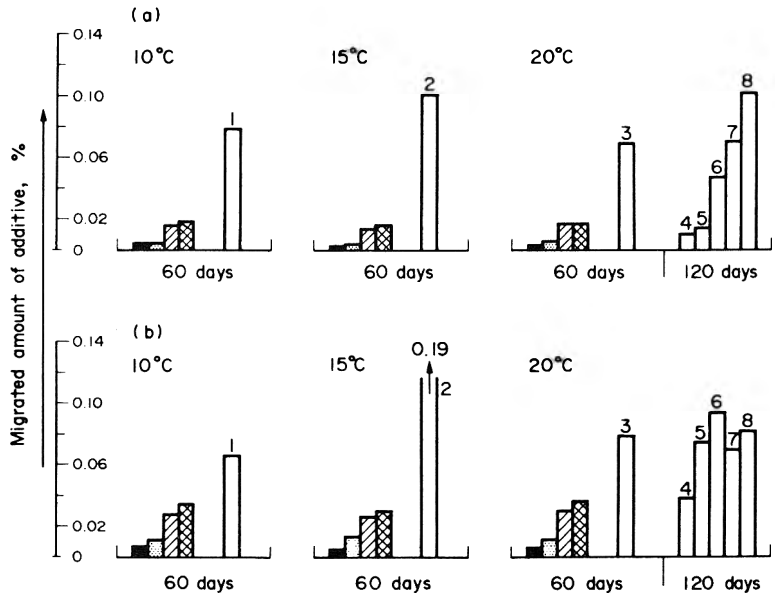


Fig. 1. Migration of (a) stearyl alcohol and (b) Irganox 17 MOK from rigid PVC into (1) fresh cheese (50% fidm), (2) margarine, (3) processed cheese (50% fidm), (4) dehydrated clear soup, (5) dehydrated thickened soup, (6) mayonnaise (80% fat), (7) mayonnaise (30% fat), and (8) orange juice and into the food simulants water (■), 3% acetic acid (▨), HB 307 (▨), and olive oil (▩). Initial levels of the additives in the polymer were 0·65% (w/w) stearyl alcohol and 1·0% (w/w) Irgastab 17 MOK and contact times were 60 days at 10, 15 or 20 C and 120 days at 20 C.

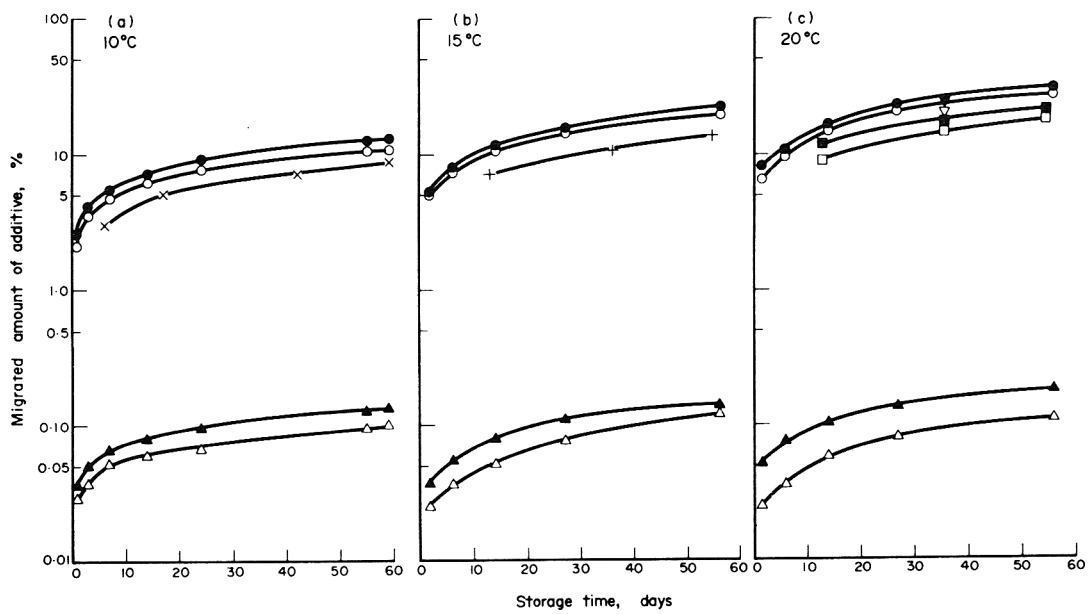


Fig. 2. Migration of BHT from HD-PE containing 0·2% (w/w) BHT into fresh cheese (×) at 10 C, into whole-milk powder (+) at 15 C, and into dehydrated soups, thickened (▼) or clear (▽) and mayonnaises containing 80% fat (■) or 30% fat (□) at 20 C, as well as into HB 307 (●), olive oil (○), 3% acetic acid (▲) and water (△) at (a) 10, (b) 15 and (c) 20 C for up to 60 days.

Migration into pharmaceutical products

Because of the wide variation in the chemical and physical characteristics of pharmaceutical products and consequently in their interactions with packaging materials, the degree of migration of low-molecular components from a plastics material will clearly depend on the type of product in contact with a given polymer. On the other hand, in the case of a single pharmaceutical product, the amount transferred is likely to vary with each type of polymer or, for any given polymer, with the mobility or volatility of the component in question. The accuracy of these predictions is confirmed by Table 3.

The amounts of stearyl alcohol and Irgastab 17 MOK transferred from rigid PVC in 10 days at 40°C were, as expected, very low; the smallest amount (for each additive, 0·004% of the amount originally incorporated into the test sheet) migrated into Ringer solution (0·94% (w/v) inorganic salts in distilled water) and the greatest (0·7% of stearyl alcohol and 0·6% of the organotin stabilizer) into a medicinal ointment containing 3% (w/w) hydroxyethylcellulose together with water (pH 6) and an unidentified pharmaceutical.

It is surprising that both rigid-PVC additives migrated not only into the liquid and gelatinous products but also, in comparable quantities, into solid products like medicinal powder and tablets. The mechanism of this type of transfer, which has been observed in other solid content/packaging material systems, is discussed later in this section.

The low migration values recorded following contact of each of the pharmaceutical products with rigid PVC demonstrates that, regardless of its physical state, none of the preparations had any significant effect on the structure of rigid PVC, which may therefore be considered a very suitable material for packing these products.

In contrast, there was a wide variation in the results obtained with the HD-PE test sheets. Thus, intense interaction between HD-PE sheets and wool wax alcohol ointment, a water–oil emulsion containing about 50% water, was indicated by the migration of 94% of the original BHT content and 75% of the Irganox 1076 during storage for 10 days at 40°C, while under the same conditions transfer of these two additives into Ringer solution was only 0·7 and 0·08%, respectively. Particularly marked was the transfer of the volatile antioxidant, BHT, from HD-PE sheets to solid pharmaceutical products, 57% of the original BHT content being found in the medicinal powder and 34 and 48% in the two types of tablets studied. The corresponding degree of transfer of the antioxidant Irganox 1076 into the same products was only some 10 or 1% of these figures, amounting to 5·1, 0·36 and 0·44%, respectively.

Since these two HD-PE test sheets differed only in the identity of the additive carrying the radioactive label, one must assume that the differences in migration behaviour were due solely to the different chemical and physico-chemical properties of the two antioxidants. The small volatile molecule of BHT is very mobile in polyolefines, while the Irganox 1076 molecule is much less mobile because of the stearyl and carboxyl groups (Fig. 3) and the compound has a much lower vapour pressure then BHT.

There was little agreement between the extent of

Table 3. *Amounts of additives transferred from test sheets to various contact media during a 16-day period of double-sided contact at 40°C*

	Migration of additives from test sheets* of							
	Rigid PVC				HD-PE			
	Stearyl alcohol†		Irgastab 17 MOK†		BHT†		Irganox 1076†	
Contact medium	%†	μg/dm²§	%‡	μg/dm²§	%‡	μg/dm²§	%‡	μg/dm²§
Metamizol Na solution (50% w/w)	0·021	2·82	0·036	8·72	7·09	241·8	0·193	3·45
Ringer solution	0·004	0·57	0·004	1·05	0·69	22·8	0·075	1·27
Medicinal ointment	0·73	103·7	0·55	124·8	5·10	186·5	0·276	4·46
Wool wax alcohol ointment (containing water)	0·010	1·33	0·032	8·09	94·1	3378·3	74·8	1265·2
Medicinal powder	0·007	1·00	0·009	2·12	56·8	2105·8	5·08	83·9
Metamizol Na tablets	0·009	1·31	0·002	0·49	34·2	1180·1	0·364	5·86
Multiple-component tablets	0·094	13·6	0·097	24·4	48·3	1823·3	0·437	7·23
Distilled water	ND	ND	ND	ND	0·48	16·8	0·065	1·15
Acetic acid (3% w/w)	0·002	0·29	0·010	2·30	0·82	29·7	0·115	2·05
Ethyl alcohol (15% v/v)	0·005	0·65	0·013	3·01	1·29	42·2	0·147	2·48
Fat simulant HB 307	0·013	1·81	0·026	6·05	89·6	2852·0	45.4	774·8
Olive oil	0·022	2·90	0·024	5·94	99·6	3392·2	53·1	871·6

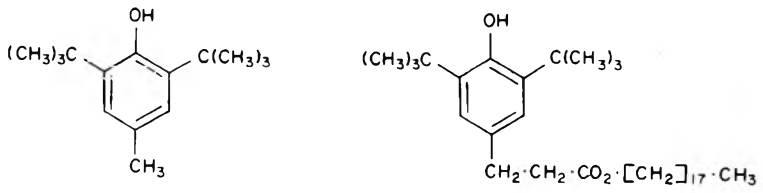
PVC = Polyvinyl chloride HD-PE = High-density polyethylene BHT = Butylated hydroxytoluene
ND = Not determined

*Test samples were 330–362 μm thick with a contact or surface area of 9·0 cm². Amounts of contact medium varied from 2·5 g (medicinal powder) to 13·7 g (aqueous Metamizol Sodium solution).

†Initial additive levels (w/w) in the polymers were stearyl alcohol 0·6%, Irgastab 17 MOK 1·0%, BHT 0·2% and Irganox 1076 (0·1%).

‡Percentage of the corrected additive content of the contact sheet (calculated from the radioactivity of the finished test foil, taking into consideration the specific radioactivity of the additive used).

§Weight of additive migrating per unit area of contact.



BHT

($C_{15}H_{24}O$; mol wt 220·4)

Irganox 1076

($C_{35}H_{62}O_3$; mol wt 530·9)

Fig. 3. Structure of BHT and Irganox 1076

migration of labelled additives from HD-PE into pharmaceutical products and that into food simulants under the same test conditions. Only in the case of Ringer solution was it possible to predict the migration from that demonstrated with distilled water or 3% acetic acid. Transfer of the additives into the medicinal ointment with a high water content or into the 50% (w/w) aqueous metamizol sodium solution was several times greater than that into aqueous food simulants.

Particular difficulties will certainly be encountered in the simulation of solid pharmaceutical preparations. Because of their different surface structures, these materials vary in their capacity to adsorb substances transferred on account of their vapour pressure from the packaging material to the 'internal air' of the pack. Figure 4 shows a simplified model of the transfer of an additive from the plastics material (I) via the gas phase (II) into the adsorbent (III). The solubilities of the additive in the packaging material and adsorbent, respectively, are demonstrated by the varying thicknesses of columns I and III, and the amount of additive (vapour pressure P_A) dissolved in the packaging material is represented by the total shaded area of column I. If transfer of the additive to the adsorbent is possible, equilibrium is reached when the solution of the additive in each medium has the same vapour pressure (P_E). The velocity of additive transfer depends on the diffusion of the additive in the packaging material and in the gas phase as well as in the adsorbent. In the model, the diffusion coefficient in the polymer is represented by the width of the aperture S.

The situations with additives characterized by low, medium and high solubility in the adsorbent are shown in parts A, B and C, respectively, of Fig. 4, the amounts transferred being indicated by the somewhat darker shading. This model shows that additives migrate not only as a result of direct contact with the packaged product but also through a gas phase.

To estimate the actual degree of contamination of pharmaceutical products with additives from the types of plastics studied from the dimensions and filling-weights of commercially available containers, the area of the packaging material required for 1 kg of product was calculated (Table 4). In the case of 1 kg

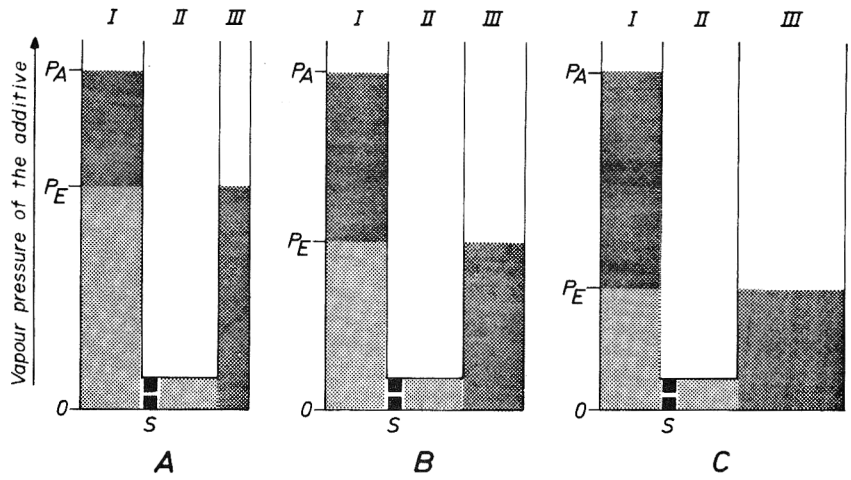


Fig. 4. Model representations of the transfer of an additive of (A) low, (B) medium and (C) high solubility in an adsorbent, from a plastics material (I) through the gas phase (II) to the adsorbent (III), the relative solubilities of the additive in the packaging material and adsorbent being indicated by the different thicknesses of columns I and III. Each representation shows the amount of additive dissolved in the packaging material (total shaded area), the amount transferred to the adsorbent (more heavily shaded area), the initial vapour pressure of the additive dissolved in the packaging material (P_A), the vapour pressure at equilibrium (P_E) and an aperture (S) representing the diffusion coefficient of the additive in the polymer.

Table 4. *Contamination of pharmaceutical products by components of rigid PVC and HD-PE packaging materials calculated from the amounts of additive migrating during storage for 10 days at 40 C*

Packed product	Packaging		Concn of additives (ppm) in pharmaceutical products packed in			
			PVC film		HD-PE film	
	Type	Area/kg of product* (dm^2)	Stearyl alcohol†	Irgastab 17 MOK†	BHT†	Irganox 1076†
Metamizol Na solution (50% w/w)	Bottle, 1 litre	5·3	0·015	0·047	1·29	0·018
	50 ml	14·0	0·040	0·122	3·39	0·048
Ringer solution	Bottle, 1 litre	5·9	0·003	0·006	0·136	0·008
Medicinal ointment	Tube, 25 g	27·2	2·82	3·40	5·07	0·121
Wool wax alcohol ointment (containing water)	Tube, 25 g	27·2	0·036	0·220	91·9	34·4
Medicinal powder	Container, 1 litre	23·2	0·023	0·049	48·9	1·95
	95 ml	56·6	0·057	0·120	119·2	4·75
Metamizol Na tablets	Blister pack‡	92·6	0·121	0·045	109·3	0·543
	Tube, 20 tablets	31·0	0·041	0·015	36·6	0·182
	Box, 250 tablets	12·3	0·016	0·006	14·5	0·072
Multiple component tablets	Blister pack‡	103·1	1·40	2·52	188·0	0·745
	Tube, 20 tablets	44·8	0·609	1·09	81·7	0·324
	Box, 100 tablets	24·7	0·336	0·603	45·0	0·179

PVC = Polyvinyl chloride HD-PE = High-density polyethylene BHT = Butylated hydroxytoluene

*Calculated from the dimensions and filling weights of conventional packs for pharmaceutical products.

†Initial additive levels (w/w) in the polymers were stearyl alcohol 0·65%, Irgastab 17 MOK 1·0%, BHT 0·2% and Irganox 1076 0·1%.

‡Individually packed tablets.

Table 5. *Amounts of additives transferred from test sheets to various cosmetic products during a 10-day period of double-sided contact at 40°C*

	Migration of additive from test sheets* of							
	Rigid PVC				HD-PE			
	Stearyl alcohol†		Irgastab 17 MOK†		BHT†		Irganox 1076†	
Contact medium	%‡	μg/dm²§	%‡	μg/dm²§	%‡	μg/dm²§	%‡	μg/dm²§
Foam bath 1	0·012	1·62	0·020	4·73	47·1	1581·7	38·9	586·4
2	0·086	11·7	0·095	21·4	77·1	2706·9	53·2	838·9
3	0·024	3·23	0·021	5·34	87·2	2788·5	59·6	906.4
Herbal shampoo	0·016	2·01	0·044	10·5	81·9	2759·8	32·8	521·5
Sulphur shampoo	0·023	3·18	0·037	8·69	78·2	2828·7	46·4	787·9
Hair cure	0·030	4·47	0·033	9·07	58·5	2066·9	0·399	6·66
Distilled water	ND	ND	ND	ND	0·48	16·8	0·065	1·15
Acetic acid (3%, w/w)	0·002	0·29	0·010	2·30	0·82	29·7	0·115	2·05
Ethyl alcohol (15%, v/v)	0·005	0·65	0·013	3·01	1·29	42·2	0·147	2·48

PVC = Polyvinyl chloride HD-PE = High density polyethylene BHT = Butylated hydroxytoluene
ND = Not determined

*Test samples were 330–362 μm thick with a contact or surface area of 9·0 cm². Amounts of contact medium varied from 10·4 (fat simulant HB 307) to 12·5 g (sulphur shampoo).

†Initial additive levels (w/w) in the polymers were stearyl alcohol 0·65%, Irgastab 17 MOK 1·0%, BHT 0·2% and Irganox 1076 (0·1%).

‡Percentage of the corrected additive content of the contact sheet.

§Weight of additive migrating per unit area of contact.

Ringer solution in a 1-litre bottle, for example, the area is only 6 dm², whereas for 1 kg multiple-component tablets, individually packed, it is of the order of 103 dm².

Even when the area/weight ratios encountered in practice are taken into consideration, the contamination of pharmaceutical products with additives from rigid PVC is very low, with maximum levels of contamination in the medicinal ointment packed in 25-g tubes. Transfer from these PVC formulations is generally so low that differences in the migration behaviour of the additives have no practical significance.

With the HD-PE formulations, however, the migration of the two additives studied depended on the type of contact medium and, particularly, on the chemical and physico-chemical properties of the additives. The practical significance of the differing behaviour of BHT and Irganox 1076 in the HD-PE test film is clear from the relative contamination of pharmaceutical products shown in Table 4. Multiple-component tablets kept for 10 days at 40°C in blister packs made from the HD-PE test sheets, for example, contained *c.*190 ppm BHT but only 0·75 ppm Irganox 1076. Clearly, the physiological safety of packaging materials made from polyolefines, polystyrene and other polymers and thus their use for foodstuffs and pharmaceutical products, will depend to a large extent on the selection of suitable additives.

Migration into cosmetics and hair-care preparations

No previous reports are available on the interaction of cosmetics materials or hair-care preparations with packaging materials, and again it is of particular interest to determine the levels of component migration and the possibility of simulating the heterogeneous products in this group by suitable test media for analytical purposes.

Exceptionally small amounts of stearyl alcohol and Irgastab 17 MOK (0·01–0·1% of the initial content for both additives) were transferred into the tested products from rigid PVC (Table 5). Assuming that 6 dm² film surface is in contact with 1 kg of the packaged product, these migration values give levels of contamination between 0·01 and 0·13 ppm (Table 6).

Transfer of BHT and Irganox 1076 from HD-PE into the same products was much greater, ranging from 47 to 88% for BHT and 0·4 to 60% for Irganox 1076 (Table 5) and giving levels of contamination for the given contact surface/weight ratio between 9 and

Table 6. *Contaminction of cosmetic and hair-care preparations by components of rigid PVC and HD-PE packaging materials, calculated from the amounts of additive migrating during storage for 10 days at 40°C*

	Concn of additives (ppm)* in cosmetic products packed in			
	PVC film		HD-PE film	
Packaged product	Stearyl alcohol	Irgastab 17 MOK	BHT	Irganox 1076
Foam bath 1	0·010	0·028	9·49	3·52
2	0·070	0·128	16·2	5·03
3	0·019	0·032	16·7	5·44
Herbal shampoo	0·012	0·063	16·6	3·13
Sulphur shampoo	0·019	0·052	17·0	4·73
Hair cure	0·027	0·054	12·4	0·40

PVC = Polyvinyl chloride
HD-PE = High-density polyethylene
BHT = Butylated hydroxytoluene

*Calculated from migration data on the assumption that 6 dm² surface area is in contact with 1 kg of product.

†Initial additive levels (w/w) in the polymers were stearyl alcohol 0·65%, Irgastab 17 MOK 1·0%, BHT 0·2% and Irganox 1076 0·1%.

17 ppm for BHT and 0·4 and 5·5 ppm for Irganox 1076 (Table 6).

A comparison of the migration data obtained for these cosmetics and hair-care preparations and for aqueous simulants (distilled water, 3% acetic acid and 15% ethanol) under identical conditions (Table 5) suggests that these are of only limited use for simulating the behaviour of the products studied.

Studies are being continued with other additives and polymers to determine the extent to which these results are typical of packaging-ingredient migration and to establish suitable test conditions and simulants for estimating the transfer of low-molecular weight components from packaging materials into foodstuffs, cosmetics and pharmaceutical preparations.

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HIGH INCIDENCE OF HEPATIC TUMOURS IN RATS FED MOULDY RICE CONTAMINATED WITH *ASPERGILLUS VERSICOLOR* CONTAINING STERIGMATOCYSTIN

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Abstract—The hepatocarcinogenicity of rice contaminated with *Aspergillus versicolor* was investigated by feeding two groups of male Donryu rats on a mouldy-rice culture added to the diet in an amount to provide 5 or 10 ppm sterigmatocystin, equivalent to a daily intake of 75 or 150 μg/rat. Of the 13 rats in each test group that died between day 465 (when the first animal with a tumour died) and day 709 (when survivors were killed), 12 of those fed 10 ppm sterigmatocystin and 11 fed 5 ppm developed hepatic tumours, mostly hepatocellular carcinomas. Three haemangiosarcomas were also observed. In view of the high incidence of hepatomas following ingestion of very low levels of sterigmatocystin, increased surveillance of this toxin in feeds and foodstuffs is recommended.

INTRODUCTION

The carcinogenic effect of sterigmatocystin, a mycotoxin produced by *Aspergillus versicolor*, *A. sydowi*, *A. nidulans* and an unidentified species of Bipolaris (van der Watt, 1974), has been well documented (Dickens, Jones & Waynforth, 1966; Fujii, Kurata, Odashima & Hatsuda, 1976; Purchase & van der Watt, 1968, 1970 & 1973; Terao, Yamazaki & Miyaki, 1973; Zwicker & Carlton, 1974). The potency of this toxin seems to be lower than that of aflatoxin B_1, but the carcinogenicity of mouldy rice contaminated with *A. versicolor* was investigated because *A. versicolor* is more frequently isolated from Japanese cereals than are aflatoxin-producers such as *A. flavus* and *A. parasiticus* (Miyaki, Yamazaki, Horie & Udagawa, 1970; Tsuruta & Manabe, 1974), and sterigmatocystin has been chemically detected in the damaged rice (Manabe & Tsuruta, 1975; Sugimoto, Minamisawa, Takano, Sasamura & Tsuruta, 1977).

Male rats of the Donryu strain were fed on a diet into which mouldy rice was mixed to provide a dietary level of 5 or 10 ppm sterigmatocystin, the lowest levels of this toxin ever to have been investigated. A similar, long-term feeding study of a rice culture of *A. versicolor* was performed by Zwicker & Carlton (1974) in mice. They reported an enhanced incidence of lung adenomas and carcinomas but not of hepatomas.

EXPERIMENTAL

Animals. Male Donryu rats purchased from Nippon Rat Co. Ltd. (Urawa) were caged in pairs in screen-bottomed cages in an air-conditioned room (20–24°C and 50–60% relative humidity) and given 15 g pelleted feed/animal/day and tap-water *ad lib.* They were 6 wk old and weighed *c.* 120 g at the beginning of the experiment.

Mouldy rice. A strain of *A. versicolor* isolated from soya bean was inoculated into minimally polished (2%) rice and cultured for 14 days at 25°C. The rice culture was air-dried at 40–50°C, powdered and subsequently kept in a cold room (3–5°C) before and after it was pelleted. The mouldy rice was mixed with a basal diet (CE-II; Clea Japan, Ltd., Tokyo) before pelleting. Chemical analysis of the mouldy rice for sterigmatocystin was carried out by gas-liquid chromatography (Manabe, Minamisawa & Matsuura, 1971). Two batches of rice culture, weighing 2·5 and 7 kg, contained 300 and 175 ppm sterigmatocystin, respectively, the analytical results obtained being the same before and after the experiment. The concentrations of the mouldy rice in the feeds were adjusted so that the feeds contained 5 or 10 ppm sterigmatocystin, involving addition of 1·7 or 3·3% in the case of the batch 1 rice culture and of 2·9 or 5·7% for batch 2.

Experimental procedure. Three groups, each of 20 rats, were fed diet containing 0 (control), 5 or 10 ppm sterigmatocystin for up to 709 days, at which time all the survivors were killed. All the animals were weighed at intervals of 2–3 wk. Those found dead as well as those killed were autopsied, and the organs were fixed with 10% neutral buffered formalin. Paraffin-embedded sections prepared from the major

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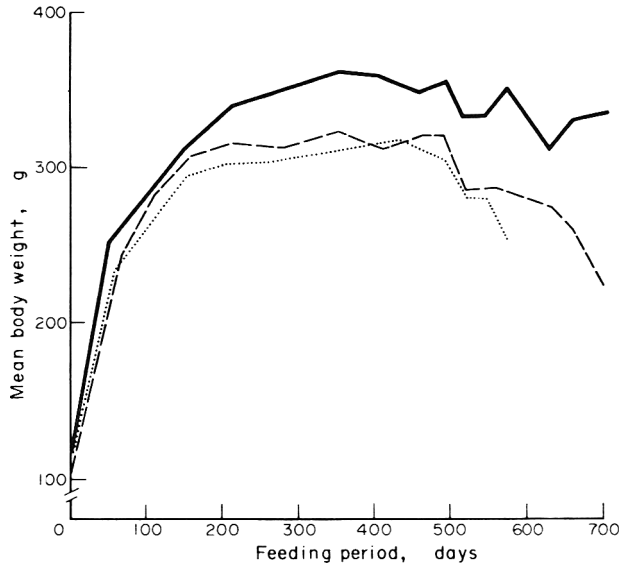

Fig. 1. Growth curves of control rats (——) and of rats fed a rice culture of *A. versicolor* providing a dietary level of sterigmatocystin of 5 (– – –) or 10 (· · · ·) ppm.

organs, including the liver, spleen, lungs and kidneys, were stained with haematoxylin and eosin, and in addition special stains (silver inpregnation and periodic acid–Schiff) were used when necessary.

RESULTS

Initially the mean body weights of the three groups (Fig. 1) showed little difference, but in the two test groups, the animals ceased to gain weight after reaching about 300 g (by day 150), while in the control group, weights continued to increase until about day 200, by which time the mean figure had reached 350 g.

About one third of the rats in the two test groups and three of the control animals died sporadically before day 460 as a result of a chronic bronchopulmonary infection. The survival rate dropped steeply between days 500 and 600 of feeding in the group on 10 ppm sterigmatocystin and between 600 and 700 days in that on 5 ppm (Fig. 2), the cause of death being hepatic tumours in about a half of the cases and chronic abscessing bronchopneumonia in the rest. Twelve of the 20 controls survived until the end of the experiment.

Hepatic tumours appeared relatively late (after day 465) in both experimental groups, but the incidence was high, being 92·3 and 84·6% in the rats surviving to day 465 in the groups on 10 and 5 ppm sterigmatocystin, respectively (Table 1). About one quarter of the animals in each group had multiple pulmonary metastases. With regard to preneoplastic lesions, all the tumour-bearing animals also had hyperplastic nodules and areas. Two animals that died on day 276 (10-ppm group) and day 435 (5-ppm group) were found to have hyperplastic nodules and areas but no carcinomas. Three of the former group and two of the latter showed only hyperplastic areas when autopsied on or after day 209.

Incidences of hepatocellular carcinoma and haemangiosarcoma are shown in Table 2. Hepatic

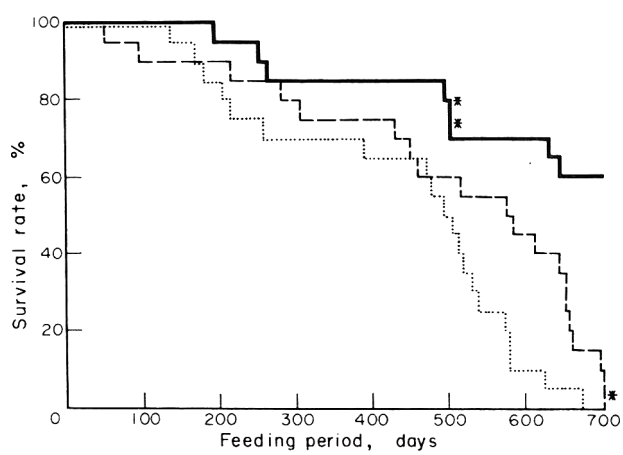

Fig. 2. Survival rate of control rats (——) and of rats ingesting 5 (– – –) or 10 (· · · ·) ppm sterigmatocystin

Table 1. *Hepatic tumours induced in male Donryu rats by a rice culture of* A. versicolor

Sterigmatocystin		No. of rats/group		No. of rats with	
Concn in feed (ppm)	Daily intake (μg/rat)	Initial	Effective*	Hepatic tumours	Lung metastases
0 (control)	—	20	17	0	0
5	75	20	13	11 (84·6%)	3
10	150	20	13	12 (92·3%)	3

*No. surviving at time of appearance of first tumour.

tumours were multiple except in one case (Fig. 3). Most of the greyish-white tumours were either well- or poorly-differentiated trabecular hepatocellular carcinomas (Fig. 4). Occasionally hepatocellular carcinomas contained areas of adenomatous or tubular pattern (Fig. 5) along with trabecular pattern. In a few cases haemorrhagic and necrotic nodules were observed. Microscopically, these tumours were identified as anaplastic haemangiosarcomas (Fig. 6). All pulmonary metastases were derived from well- or poorly-differentiated hepatocellular carcinomas.

Centrilobular atrophy of the hepatocytes, large basophilic hepatocytes in the periportal regions, and scanty single-cell necrosis were found in the non-tumorous portion of the liver, especially in animals that died between 200 and 450 days, i.e. shortly before the appearance of the first tumour. Bile-duct proliferation, with or without fibrosis, was seen occasionally after 400 days in both the experimental rats and in the controls killed at termination.

A relatively large number of animals contracted pulmonary infections, notably varying degrees of mild bronchitis, focal and extensive chronic bronchopneumonia, frequently with bronchiectasis, and pulmonary abscesses (Table 3). The last was the cause of death in two of the three controls that died after 496 days, and in half of the tumour-bearing rats in the test groups.

The main findings in other organs were a minute adrenal cortical adenoma in one control rat and a unilateral ischaemic necrosis of the kidney in another control. Atrophy of the spleen and varying degrees of inhibition of spermatogenesis often occurred in rats of all groups. No intestinal tumours were found in any animals.

DISCUSSION

Ingestion of the carcinogenic mycotoxin, sterigmatocystin, seems likely to be a common occurrence in Japan, for several reasons. Firstly *A. versicolor*, which produces sterigmatocystin, is a common contaminant of domestic cereals (Miyaki *et al.* 1970; Tsuruta & Manabe, 1974). In contrast, strains of *A. flavus* and *A. parasiticus*, which produce aflatoxins, have been isolated mainly from imported staples (Ichinoe & Kurata, 1976; Tsuruta, Sugimoto, Minamisawa & Manabe, 1974). Secondly, contamination of warehouse-stored rice with sterigmatocystin has been demonstrated by chemical analysis. Although present only in the mouldy rice which is unsuitable for consumption, its concentration reached 16·3 ppm

Table 2. *Histological classification of hepatic tumours induced in rats by a rice culture of* A. versicolor

Dietary level of sterigmatocystin (ppm)	No. of rats with					
	All tumours	Adenoma	Hepatocellular carcinoma			Haemangio sarcoma
			Well differentiated	With tubular pattern	Poorly differentiated	
0	0	0	0	0	0	0
5	11	2	9	6	5	1
10	12	4	12	5	3	2

Table 3. *Occurrence of pulmonary infections in untreated rats and in those fed a rice culture of* A. versicolor

Dietary level of sterigmatocystin (ppm)	No. animals examined	No. of animals with		
		Abscessing bronchopneumonia	Chronic bronchopneumonia	Chronic bronchitis
0	18	5	1	6
5	17	8	5	1
10	18	8	2	4

(Manabe & Tsuruta, 1975). Moreover, where there is simultaneous contamination with several fungal species, *A. versicolor* often predominates (Tsuruta & Manabe, 1974) and its production of sterigmatocystin remains relatively unimpaired (Sugimoto *et al.* 1977). Recently, species of the *A. glaucus* group (notably *A. chevalieri*, *A. ruber* and *A. amstelodami*) were reported to produce sterigmatocystin (Schroeder & Kelton, 1975). As these are also common contaminants of Japanese cereals (Saito, Ishiko, Enomoto, Ohtsubo, Umeda, Kurata, Udagawa, Taniguchi & Sekita, 1974; Saito, Ohtsubo, Umeda, Enomoto, Kurata, Udagawa, Sakabe and Ichinoe, 1971; Tsuruta & Manabe, 1974), the importance of sterigmatocystin in the field of food hygiene is further emphasized.

The analysis of sterigmatocystin and its derivatives in cereals still presents some problems in the clean-up stage (T. Hamasaki, personal communication 1977). Nevertheless, in the present experiment, the level of sterigmatocystin in the diet was monitored by gas-liquid chromatography (Manabe *et al.* 1971; Manabe, Minamisawa & Matsuura, 1973). Unfortunately there is, as yet, no reliable bioassay method for sterigmatocystin to compare with the duckling test for aflatoxins (Newberne, Wogan, Carlton & Abdel-Kader, 1964).

Usually sterigmatocystin is the major metabolite of *A. versicolor*, but the rice culture used in this study may have contained bisfuranoid derivatives other than sterigmatocystin (van der Watt, 1974). One strain of *A. versicolor* produces as much 5-methoxy-sterigmatocystin as sterigmatocystin (T. Hamasaki, personal communication 1977). Chemical analysis of the mould-infected rice used in this experiment is now being extended to cover other sterigmatocystin derivatives.

A recent study on the mutagenic activity of sterigmatocystin and its derivatives using the Ames' system revealed a stronger mutagenic potency in sterigmatocystin than among the related substances (Nagao, Honda, Hamasaki, Natori, Ueno, Yamazaki, Seino, Yahagi & Sugimura, 1976). It may be reasonable, therefore, to consider that the dietary level of sterigmatocystin itself is the most important factor in the carcinogenic effect of *A. versicolor*.

This study has shown that rice-culture diets containing sterigmatocystin in concentrations as low as those that may occur under natural conditions (Manabe & Tsuruta, 1975) cause a high frequency of hepatic tumours in rats—92·3 and 84·6% with 10 and 5 ppm, respectively. The latent period was around 470 days. These hepatic-tumour incidences were as high as that demonstrated in an earlier study (Purchase & van der Watt, 1970), but the latent period was longer than in the 1970 test, probably because different strains of rat were used. Purchase & van der Watt (1970) used a sub-line of Wistar rat, while we used the Donryu strain. The susceptibility of these strains to sterigmatocystin differed widely in acute toxicity tests (Kimura, 1976; Saito, Ueno & Kimura, 1975), the single lethal dose of sterigmatocystin being less than 500 mg/kg in Wistar rats but more than 2000 mg/kg in the Donryu strain. Another reason for the delayed appearance of tumours may have been that the animals were older at the onset of feeding in the current experiment than in the earlier one. It should be emphasized, however, that the high incidence of hepatoma was observed with a dose level as low as 5 ppm, or 75 μg/animal/day, even in the strain of rat most resistant to sterigmatocystin.

There was no significant difference in tumour incidence at the two dose levels used. The high incidence recorded suggests that sterigmatocystin would induce hepatic tumours at even lower doses. In fact, hepatic tumours were found to have developed in two out of six Fischer rats after they had been fed for 40 wk on mouldy rice infected with *A. versicolor* and containing only 1 ppm sterigmatocystin (Enomoto, Mabuchi, Miyata, Naoe, Takada & Yamazaki, 1977). The susceptibility of different rats to aflatoxin B_1 also seems to vary. Wogan & Newberne (1967) reported that 0·015 ppm aflatoxin B_1 caused a high incidence of hepatoma in 103 wk, while, according to Ward, Sontag, Weisburger & Brown (1975), a concentration as high as 2 ppm induced hepatomas in only 75% of another group of rats in 65 wk. If a similar situation existed for sterigmatocystin, the effects induced by the 5-ppm dietary level in the relatively resistant Donryu strain would probably occur with levels of less than 1 ppm in more susceptible strains.

As for the pathological findings in our study, the hepatic tumours were frequently associated with hyperplastic nodules and areas and multiple tumours were observed in nearly all the livers. However, there was no liver cirrhosis. Even with sterigmatocystin doses as high as 100 or 200 ppm, cirrhosis has never been reported in conjunction with the hepatomas (Kimura, 1976; Purchase & van der Watt, 1970). The hepatocellular carcinomas were well- or poorly-differentiated trabecular hepatomas. Of particular interest were frequent foci of a tubular pattern in the hepatoma nodules, found in 11 of 23 animals. The tumour cells of this area were reported to show both hepatocellular and cholangiocellular characteristics when studied by electron microscopy (Terao, 1977). Pulmonary metastasis of the carcinomas was fairly common, but there was no peritoneal dissemination in any rat, even when the tumours were relatively large and characterized by necrosis and haemorrhage. Three cases of haemangiosarcoma were also combined with hepatocellular carcinomas, but none of these showed metastases.

It may be concluded that sterigmatocystin is a more potent carcinogen than was previously thought, having nearly the same degree of carcinogenicity as aflatoxin B_1. Although it has only been detected in obviously damaged rice, a more extensive survey of the presence of sterigmatocystin and related compounds in staple foods such as rice is recommended.

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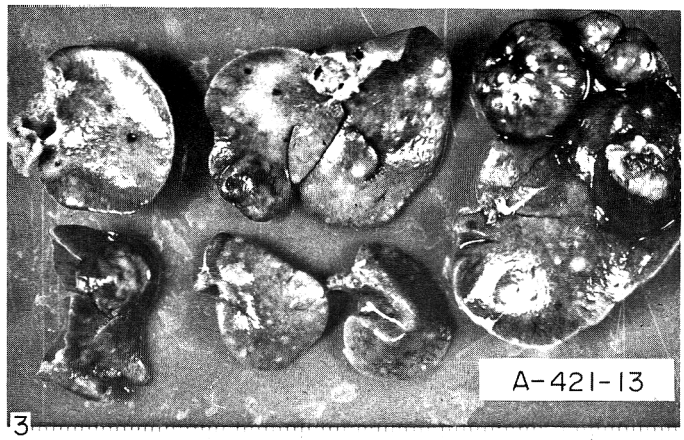

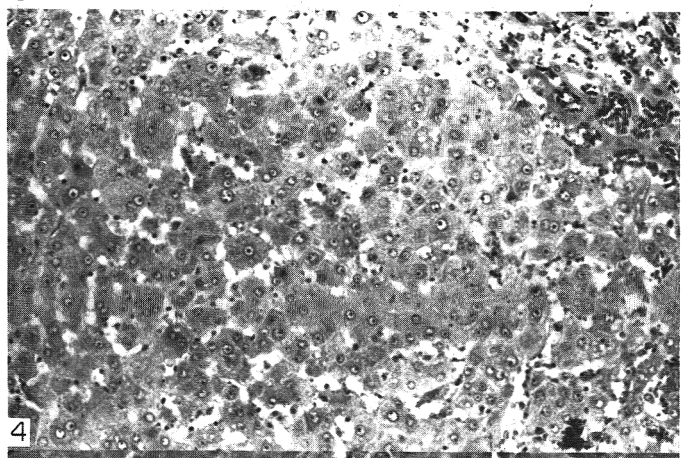

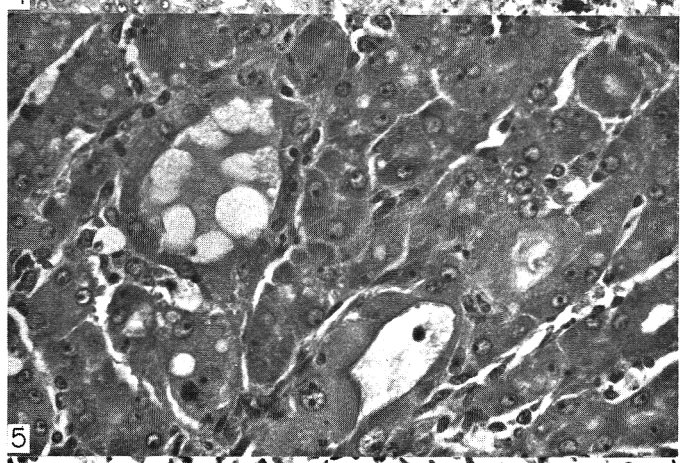

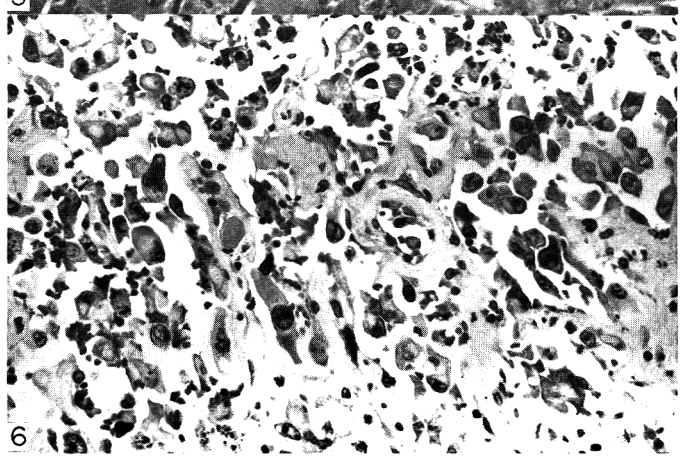

Fig. 3. Macroscopic view of the liver of a rat fed 5 ppm sterigmatocyst n, showing multiple nodules of hepatocarcinoma in the left lateral lobe. Small hyperplastic nodules are scattered throughout the lobes.

Fig. 4. Part of a well-differentiated trabecular hepatocarcinoma.

Fig. 5. Well-differentiated hepatocarcinoma with areas of adenomatous structure.

Fig. 6. A representative section of anaplastic angiosarcoma.

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EFFECT OF DIETARY INDOLE-3-CARBINOL ON THE INDUCTION OF THE MIXED-FUNCTION OXIDASES OF RAT TISSUE

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Abstract—Indole-3-carbinol (IC), a compound present in cabbage and other cruciferous vegetables, was fed for 3 wk to young male Sprague–Dawley rats at dietary levels of 50, 500, 5000 and 7500 ppm. Because lower body weights were recorded in the animals fed the two highest levels of IC, pair-fed control groups of rats were used in a second experiment. Increased relative liver weights with enhanced activities of hepatic aminopyrine-*N*-demethylase, *p*-nitroanisole *O*-demethylase and aryl hydrocarbon hydroxylase (AHH) accompanied by higher cytochrome *P*-450 levels were observed only in the rats fed the two highest levels of IC either *ad lib.* or in the paired-feeding study. AHH activity increased also in the kidney, with enhanced glucuronide excretion, in the rats fed either of the two highest levels of IC. Intestinal AHH exhibited a significant induction only as a result of the 7500-ppm treatment. The dose-response relationships differed among the tissues. The induction of tissue mixed-function oxidases by only extremely high dietary levels of IC supports the conclusion that this indole is not the specific vegetable constituent that induces these enzymes in rats fed Cruciferae.

INTRODUCTION

The tissue enzymes known collectively as microsomal enzymes or mixed-function oxidases (MFOs) are well known for their capacity to metabolize drugs, pesticides and other synthetic chemicals to more hydrophilic compounds (Conney, 1967; Parke, 1968). Animal feeding studies have demonstrated MFO induction with common vegetables, especially those from the botanical family, Cruciferae, such as cabbage, cauliflower and Brussels sprouts (Babish & Stoewsand, 1975) and especially with the intestinal aryl hydrocarbon hydroxylase (AHH) system (Wattenberg, 1971).

A number of indoles occur in cruciferous plants (Jones & Taylor, 1957) as a result of the enzymatic hydrolysis, by myrosinase, of indolylmethyl glucosinolate, which is present in significant amounts (Virtanen, 1965). Four indoles have shown an inducing effect on AHH in the small intestine and liver of the rat, with indole-3-carbinol (IC) producing the greatest enzyme elevation (Loub, Wattenberg & Davis, 1975). Recently, it was observed that rats fed dried Brussels sprouts or cabbage increased the *in vitro* intestinal metabolism of hexobarbitone, phenacetin, 7-ethoxycoumarin and benzo[*a*]pyrene. Pretreatment of the animals with indoles, expecially indole-3-carbinol, also stimulated intestinal drug metabolism, but to a smaller extent than did the feeding of cruciferous vegetables (Pantuck, Hsiao, Loub, Wattenberg, Kuntzman & Conney, 1976).

The purpose of the present investigation was to observe the effects of increasing dietary levels of IC on intestinal, renal and hepatic AHH activities as well as on hepatic aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase and cytochrome *P*-450 levels. Urinary glucuronide production was also measured in rats fed high levels of IC.

EXPERIMENTAL

Materials and diets. Incorporation of IC (Aldrich Chemical Co., Milwaukee, WI) into a purified 'AIN 76' diet (Bieri, Stoewsand, Briggs, Phillips, Woodard & Knapka, 1977) at levels of 50, 500, 5000 and 7500 ppm was accomplished by dissolving the required amount of test material in 300 ml acetone and adding the solution to 6 kg of the diet. The diet was then mixed at high speed until all odour of acetone disappeared. The control diet was mixed with 300 ml acetone without the IC in an identical manner.

Animals. Groups of six male Sprague–Dawley weanling rats (ARS, Sprague–Dawley, Madison, WI) were fed the five diets *ad lib.* for 3 wk. The animals were weighed and then killed by rapid decapitation, between 6.00 and 7.00 hr following an 8-hr fast, and tissue-MFO activity, hepatic microsomal protein and cytochrome *P*-450 levels were compared in the differently treated groups. Since the mean body weights of the two groups of rats fed the two highest levels of IC were relatively low, another experiment was run with two groups of rats on the control diet, pair-fed to the mean dietary intake of animals fed either 5000 or 7000 ppm IC. MFO activities and urinary excretion of glucuronide were determined in the pair-fed rats placed on these dietary treatments.

Preparation of tissue homogenates. Livers were perfused *in situ* with cold 0·9% NaCl solution, weighed, sliced and homogenized in 4 vols ice-cold 1·15% KCl containing 20 mM-Tris HCl buffer (pH 7·4) at 37°C, using a Potter-Elvehjem teflon glass homogenizer fitted to a mechanical drill. Sampling of intestinal tissue involved scraping the mucosa of a 20-cm length of the small intestine with a scapel blade and suspending

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the cells in 5 ml of the buffer. Preparation of homogenates of kidney and other tissues (adrenal, brain, cardiac and testicular tissues) involved simply weighing, slicing and homogenizing the tissue in 9 vols KCl-Tris buffer to obtain a 10% (w/v) suspension for centrifugation. All microsomal-enzyme analyses were performed using the 12,000 **g** supernatant after it had been decanted through glass wool. For quantitation of cytochrome *P*-450, this supernatant was centrifuged at 105,000 **g** twice after resuspension of the pellet in 40 mM-Tris-HCl buffer (pH 7·4) at 0–4°C.

Incubation mixtures. A 2-ml incubation mixture contained 1 ml of the supernatant, 0·15 M-KCl, 20 mM-Tris HCl buffer (pH 7·4), 1·2 mM-NADP, 9·8 mM-isocitrate, 6·0 mM-$MgCl_2$, 7·5 mM-semicarbazide HCl, when assaying *N*-demethylation, 0·18 units isocitrate dehydrogenase and substrate in one of the following concentrations: aminopyrine, 7·6 mM, benzo[*a*]-pyrene, 100 μM, and *p*-nitroanisole, 200 μM. This mixture was incubated under ambient air in 30-ml beakers using a Dubnoff shaking incubator at 37°C. All observations were made during a time interval when reaction rates were linear. Reactions were initiated by addition of the substrate and were terminated with either 1 ml 20% trichloroacetic acid solution or 2 ml ice-cold acetone.

Reaction-product measurements. The activity of *N*-demethylase was measured by following aminopyrine generation of formaldehyde using a reagent containing 2 M-ammonium acetate, 50 mM-acetic acid and 20 mM-acetylacetone (Nash, 1953). The reaction catalysed by the AHH system with benzo[*a*]pyrene as the substrate was followed by determining fluorometrically the amount of 3-hydroxybenzo[*a*]pyrene formed (Nebert & Gelboin, 1968). Microsomal *p*-nitroanisole *O*-demethylase activity was determined by measuring the *p*-nitrophenol product (Kato & Gillette, 1965). Quantitation of cytochrome *P*-450 was by the dithionite–carbon monoxide method (Schoene, Fleischmann, Remmer & Oldershausen, 1972) in pellets suspended between 0·1 and 0·5 mg protein/ml (Sutherland, Cori, Haynes & Olsen, 1949) using crystalline bovine serum albumin as standard.

Urinary glucuronide determination was accomplished by adding 2 ml 0·35% naphtharesorcinol, 3 ml 15 N-H_2SO_4 and 0·2 ml 1% chloramine T to 2 ml of a 1:500 dilution of urine. This mixture was heated for 2 hr in a boiling-water bath, cooled and extracted with 10 ml water-saturated ethyl acetate. This layer was removed and a quantitative spectrophotometric determination was carried out at 565 nm.

Statistical procedures. Analysis of variance, comparisons of mean values and correlation-coefficient determinations used procedures described by Steel & Torrie (1960).

RESULTS

Experiment 1

Rats consuming IC at either the 5000 or 7500-ppm level gained significantly less body weight and showed a concomitant increase in relative liver weight (Table 1). Although food wastage was a problem, especially in the IC-supplemented diets, an average of 15·8–19·0 g/rat/day was consumed in all the treated groups.

As shown in Table 1, the amount of hepatic microsomal protein was significantly increased in the rats fed the highest level of IC. The quantities of cytochrome *P*-450 were essentially 100 and 200% higher than the control value in the 5000- and 7500-ppm groups, respectively.

The hepatic microsomal-enzyme response to the feeding of IC is graphically represented in Figs 1a–c, and follows the pattern of increased relative liver weights and increased cytochrome *P*-450 content. Hepatic AHH activity increased linearly with the log IC in the diet, but only the two highest levels of IC supplementation produced significantly elevated levels of hydroxylating activity. Although the relative levels of induction differ for hepatic AHH and *N*- and *O*-demethylases, the pattern of induction is essentially the same, with the two highest levels of IC producing significantly increased levels of enzyme activity (Figs 1b,c). Linear correlations of activities versus the log of dietary IC are 0·97, 0·94 and 0·94 for hepatic AHH and *N*- and *O*-demethylases, respectively.

Renal AHH was significantly higher in the groups on the two highest levels of IC, as depicted in Fig. 1d, the relationship between dietary IC and renal AHH being linear rather than logarithmic. Intestinal AHH showed significant induction only at the highest level of IC supplementation (Fig. 1e).

No increase in AHH activity was detected in adrenal, brain, cardiac or testicular tissue from rats fed diets containing IC.

Experiment 2

The results of the paired-feeding study are shown in Table 2. No differences were observed in the final body weights of pair-fed animals, indicating that IC had no effect on food efficiency. Relative liver weights

Table 1. *Growth, relative liver weight, hepatic microsomal protein content and cytochrome* P-*450 concentration of male rats fed 50–7500 ppm indole-3-carbinol in the diet for 3 wk*

Dietary level (ppm)	Terminal body weight (g)	Relative liver weight (% of body weight)	Microsomal protein (mg protein/g liver)	Cytochrome *P*-450 (nmol/mg microsomal protein)
0	188·7 ± 8·7	4·4 ± 0·2	12·05 ± 1·21	1·13 ± 0·12
50	202·0 ± 9·3	4·8 ± 0·1	11·06 ± 1·53	1·16 ± 0·17
500	200·1 ± 11·3	4·9 ± 0·3	13·07 ± 1·51	1·42 ± 0·14
5000	159·6 ± 7·9**	7·2 ± 0·2*	16·34 ± 1·82	2·84 ± 0·07**
7500	110·6 ± 8·7**	7·8 ± 0·4*	20·23 ± 1·08†	3·59 ± 0·20**

Values are means ± SEM for groups of six rats and those marked with asterisks differ significantly from the control value: *$P < 0·05$; **$P < 0·01$.

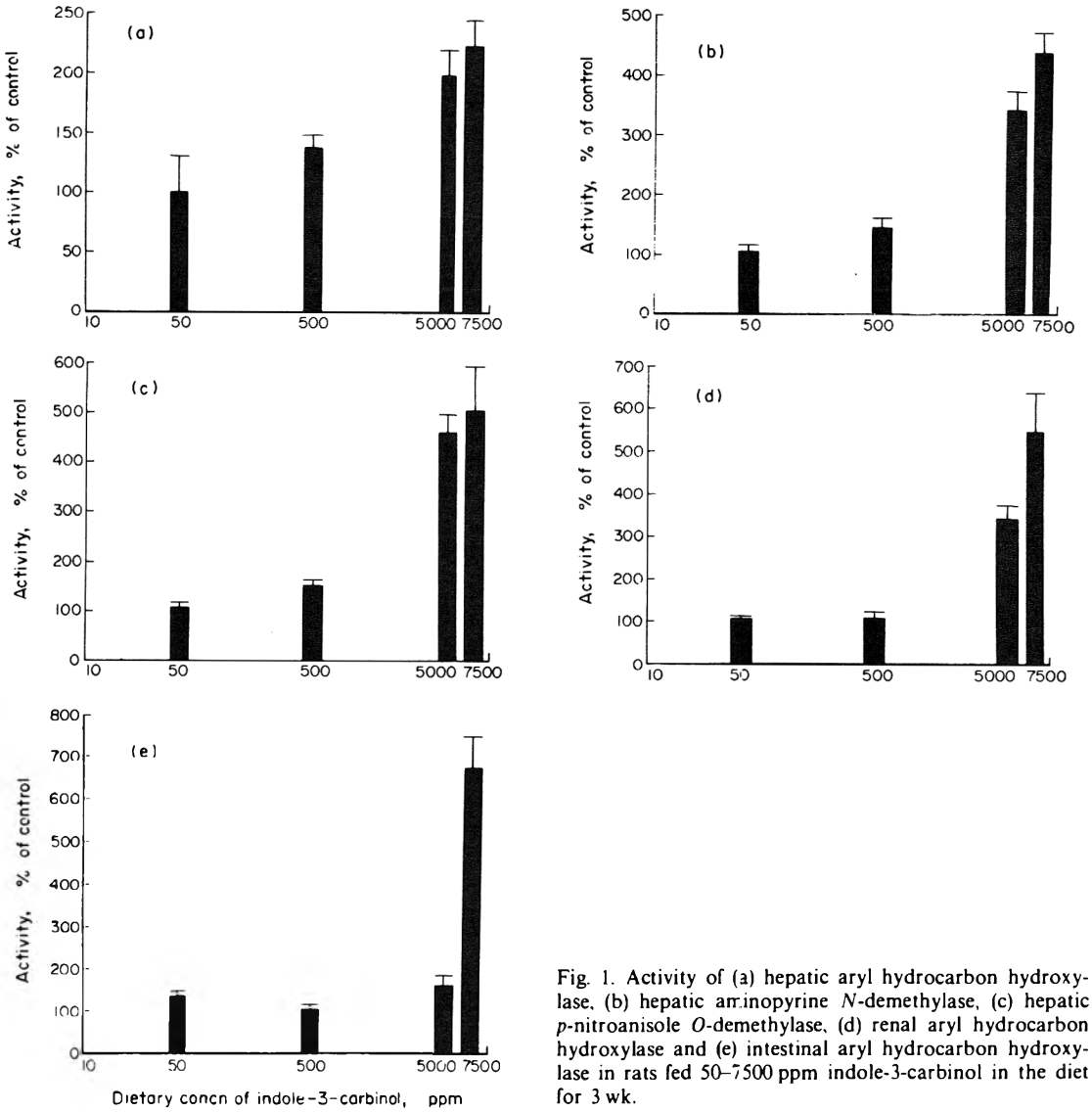

Fig. 1. Activity of (a) hepatic aryl hydrocarbon hydroxylase, (b) hepatic aminopyrine *N*-demethylase, (c) hepatic *p*-nitroanisole *O*-demethylase, (d) renal aryl hydrocarbon hydroxylase and (e) intestinal aryl hydrocarbon hydroxylase in rats fed 50–7500 ppm indole-3-carbinol in the diet for 3 wk.

Table 2. *Effect of high dietary levels (5000 or 7500 ppm) of indole-3-carbinol on body weights and mixed-function oxidases of pair-fed rats*

Parameter	Dietary level (ppm)...	Values (means ± SEM) for groups of six rats			
		5000	0†	7500	0†
Body weight (g)		191·8 ± 10·4	202·8 ± 8·9	169·1 ± 11·4	176·8 ± 7·2
Aryl hydrocarbon hydroxylase activity (pmol product/mg protein/hr)					
Hepatic		17581 ± 1557*	10674 ± 2009	15607 ± 1446*	9013 ± 1022
Intestinal		108 ± 30	62 ± 27	148 ± 34*	62 ± 25
Renal		1016 ± 188*	335 ± 77	1078 ± 178*	280 ± 58
Hepatic aminopyrine *N*-demethylase (nmol product/mg protein/hr)		86·6 ± 5·9*	21·0 ± 2·5	96·4 ± 9·3*	22·4 ± 1·6
Hepatic *p*-nitroanisole *O*-demethylase (nmol product/mg protein/hr)		9·5 ± 0·9*	5·1 ± 0·5	9·9 ± 0·7*	5·2 ± 0·6
Hepatic cytochrome *P*-450 (nmol/mg microsomal protein)		1·75 ± 0·11*	0·54 ± 0·05	2·04 ± 0·27*	0·70 ± 0·06

Values marked with an asterisk differ significantly from that for the pair-fed control: $*P < 0·05$.

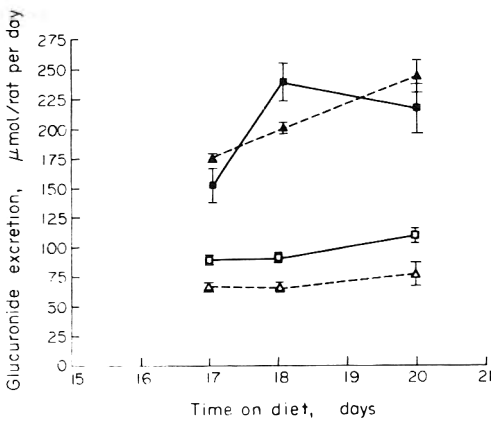

Fig. 2. Urinary excretion of glucuronide by rats fed diet containing 5000 ppm indole-3-carbinol (■——■) and by their pair-fed controls (□- - - -□) and by those fed 7500 ppm (▲- - -▲) or a pair-fed control diet (△- - -△).

were significantly increased, as in the first experiment, at the 5000- and 7500-ppm levels.

AHH activities of the liver, intestine and kidney all increased significantly in the 7500-ppm IC group, compared with the pair-fed controls, and a similar induction was observed in the 5000-ppm group, except in the case of intestinal AHH, the increase in which was not statistically significant. The activities of aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase were increased in the livers of the animals fed the test diets by about 320 and 88%, respectively. IC administration increased the cytochrome *P*-450 content of the liver by about 200%.

Figure 2 shows the daily urinary glucuronide excretion during the final week of the paired feeding. Both dietary levels of IC increased total urinary glucuronide output, the totals reached being the same in each case. However, the control group pair-fed to the 7500-ppm IC group had a consistently lower daily glucuronide output than the control group pair-fed to the 5000-ppm group (67 *v.* 92 μmol).

DISCUSSION

An increase in relative liver weight is generally taken as *prima facie* evidence of enzyme induction (Gilbert & Golberg, 1965), the enlargement being assumed to be an adaptation to an increased functional load. Hepatic MFO activity, cytochrome *P*-450 and relative liver weights all increased when 5000 or 7500 ppm IC was added to the rat diet. Lower levels of dietary IC were not effective.

Dose-response relationships varied among the tissues. The patterns of enhanced MFO induction in the liver were generally similar regardless of the enzyme substrate. Renal AHH (Fig. 1d) was significantly increased at the two highest levels of IC supplementation but, in contrast to the situation in the liver, the relationship between the dietary level of IC and renal AHH appeared to be linear rather than logarithmic. The correlation coefficient was 0·99 for a linear relationship and only 0·87 for a logarithmic correlation. As seen in Fig. 1e and Table 2, intestinal AHH only showed significant induction in animals fed the highest level of IC supplementation. This relationship between dietary IC and AHH induction appeared to be neither logarithmic nor linear, but possibly third order with respect to a threshold and maximum concentration of IC in the diet. Adrenal, brain, cardiac, and testicular tissues exhibited no increase in AHH activity with dietary IC.

An increase in hepatic cytochrome *P*-450 was seen in the rats fed the highest levels of IC but no differences were observed in the ratio of cytochrome *P*-450–CO difference spectra to the sum of type *a* and type *b* cytochrome as determined by *n*-octylamine binding (Jefcoate, Calabrese & Gaylor, 1970).

The enhanced urinary excretion of glucuronide by rats fed high -IC diets indicates the well-known synthesis of uridine diphosphate glucuronic acid for donation of the glucuronic acid moiety for combination with a reactive group or IC to promote rapid elimination (Norton, 1975).

From calculations based on the data of Josefsson (1967) it can be predicted that the indole-containing thioglucosides, glucobrassicin and neoglucobrassicin, of Cruciferae would constitute about 500 ppm of the dried plant material. Allowing for complete conversion of glucobrassicin to IC by myrosinase, the daily intake would be no more than 10 mg/rat when the diets were supplemented with 25% of the dried vegetable (Babish & Stoewsand, 1975; Loub *et al.* 1975). The present study indicated that the consumption of diets containing 500 ppm or less of IC had no effect on tissue MFO, cytochrome *P*-450 or relative liver weights; only when relatively massive dietary levels of IC were present, were these parameters increased in a manner reflecting a direct response to dose. Specific dosing of pure indoles twice daily for 3 days, followed by measurement of enzyme induction (Loub *et al.* 1975) or drug metabolism (Pantuck *et al.* 1976) 18 hr after the final dose, does not duplicate the situation of feeding vegetable-containing diets for 1–3 wk. It appears unlikely, therefore, that IC is the specific compound responsible for the increased tissue-MFO activity found in animals fed cruciferous plants. However, this is not to say that IC could not be one of the natural food chemicals contributing to this phenomenon.

Induction of MFO is known both to function as a detoxification system and to promote the formation of more toxic derivatives, as in the AHH activation of benzo[*a*]pyrene to more carcinogenic metabolites (Gelboin, Kinoshita & Wiebel, 1972). It appears crucial to expand our knowledge on the natural food compounds that may normally affect this fundamental biological process.

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ON THE AETIOLOGY OF SCOMBROID POISONING: CADAVERINE POTENTIATION OF HISTAMINE TOXICITY IN THE GUINEA-PIG

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Abstract—The peroral toxicity of histamine in the guinea-pig is shown to be potentiated by simultaneous administration of cadaverine. Toxic effects are observed with relative cadaverine–histamine levels that are similar to those found in scombrotoxic fish. On the basis of currently available evidence, it seems likely that cadaverine, along with histamine, is of importance in the aetiology of scombroid poisoning.

INTRODUCTION

Sporadic outbreaks of human poisoning have occurred following ingestion of scombroid fish, such as bonito, mackerel and tuna. Documented outbreaks, usually involving small numbers of individuals, have been reported in the United States since the late 1930s (Brown & Arnold, 1978), but in 1973 there occurred an outbreak in which 254 people were affected (*Morbidity and Mortality*, 1973). Histamine levels are invariably elevated in the toxic fish and the signs of poisoning, including nausea, vomiting, facial flushing, headache, thirst and gastric pain, are similar to those of histamine poisoning (Halstead & Courville, 1967). Whereas there seems to be little doubt that histamine is involved in scombroid poisoning, this compound does not appear to be the only causative agent. Histamine exhibits low toxicity on peroral administration to man (Weiss, Robb & Ellis, 1932). In addition, human consumption of tuna spiked with histamine in amounts that cause poisoning when present in toxic fish causes no symptoms of toxicity (Kimata, 1961). The role of histamine in poisoning from fish products has been discussed in a recent informative review (Brown & Arnold, 1978).

Efforts to isolate and identify histamine-like substances, notably saurine, from toxic fish have not been successful (Foo, 1976; Olcott & Lukton, 1961). Such substances do not appear to play a general role in fish poisoning.

Several compounds have been reported to potentiate the biological activity of histamine in laboratory animals. Of these substances, putrescine and cadaverine occur in appreciable quantities in toxic fish and in low levels in non-toxic fish (Kim, 1978; Mietz, 1977). Effects of these substances on histamine reactions have been observed in the guinea-pig or its tissues. Parrot & Nicot (1966) have shown that the peroral toxicity of histamine in this animal is increased by a factor of ten when the histamine is administered 40 min after oral administration of putrescine. The putrescine dosage used was five times that of histamine on a weight basis, and potentiation was not observed on simultaneous administration of the two compounds. Arunlakshana, Mongar & Schild (1954) observed pronounced potentiation by cadaverine of histamine-induced reactions *in vitro* in the guinea-pig ileum, trachea and uterus, and Akamatsu, Kongo & Matsuoka (1964) and Mongar (1957) observed that both putrescine and cadaverine potentiated histamine-induced contractions in the guinea-pig ileum. Studies of the potentiation effects of cadaverine in whole animals have not been reported.

Recent studies have shown that the levels of cadaverine in toxic or decomposed fish are generally several times greater than the levels of putrescine (Kim, 1978; Mietz, 1977). These data and the fact that potentiation in the guinea-pig was observed only with relatively high doses of putrescine administered prior to histamine (Parrot & Nicot, 1966) suggest that putrescine may not play a central role in histamine poisoning from fish. Cadaverine, however, may be an important factor. We report here some observations on cadaverine potentiation of histamine toxicity in the guinea-pig.

EXPERIMENTAL

Aqueous solutions of the dihydrochlorides of putrescine and cadaverine (Calbiochem, La Jolla, CA) and histamine (Sigma, Chemical Co., St. Louis, MO) were administered by stomach tube in volumes not exceeding 20 ml/kg body weight to groups of female guinea-pigs weighing 350–450 g. Solutions were administered at a rate of 2 ml/min through a no. 8 French catheter attached to a 10-ml syringe. Animals were housed individually and fasted for 24 hr prior to each experiment. Survival rates were tabulated for the subsequent 24 hr.

Five different experiments were carried out, involving administration of various doses of cadaverine or putrescine and/or histamine, as outlined in Table 1, to groups of five to ten guinea-pigs.

RESULTS AND DISCUSSION

Characteristic reactions to toxic histamine-containing solutions included gasping respiratory movements followed by convulsions. Autopsies often revealed

Table 1. *Lethal effects of amines in the guinea-pig*

Experiment no.	Treatment: Amines*	Doses (mg/kg)	No. deaths/group†
I	Cadaverine/histamine	15/150	0/6 (0)
		50/150	3/9 (33)
		75/150	5/6 (83)
		100/150	8/9 (89)
		150/150	6/6 (100)
II	Cadaverine	500	0/5 (0)
		1000	2/5 (40)
		1500	6/6 (100)
III	Histamine	150	0/5 (0)
IV	Cadaverine/histamine	150/50	2/6 (33)
	Putrescine/histamine	150/50	0/6 (0)
V	Cadaverine/histamine (40-min interval)	100/150	6/10 (60)

*Administered as the dihydrochloride in aqueous solution.
†No. of deaths/no. of animals treated (expressed as a percentage in parenthesis).

perforation of the gastric wall. Reactions to toxic doses of cadaverine were similar, varying mainly in degree.

The effects of cadaverine on peroral histamine toxicity in the guinea-pig are presented in Fig. 1. The data for these and other experiments to be discussed below are presented in Table 1. Oral administration of cadaverine dihydrochloride by itself revealed a relatively low toxicity in the guinea-pig, with an LD_{50} of about 1000 mg/kg and a relatively high minimum lethal dose (MLD) of more than 500 mg/kg. In our studies, a histamine dose of 150 mg/kg, the LD_{10} level determined by Parrot & Nicot (1966), caused no deaths in the test group. However, simultaneous administration of histamine (150 mg/kg) with relatively low doses of cadaverine led to a marked increase in toxicity. A dose of 150 mg histamine/kg and 50 mg cadaverine/kg killed 33% of the group, a figure increased to 84% by a rise in cadaverine dose to 75 mg/kg.

This effect cannot be accounted for by simple addition of toxicities, a point illustrated by Fig. 2, in which the oblique line indicates the various combination of doses that would be required to give a 50% death rate if the toxicities were additive. Thus, this hypothetical LD_{50} would be achieved with histamine and cadaverine doses of 150 and 400 mg/kg, respectively. This cadaverine dose is approximately eight times the level actually required.

The potentiation effects of cadaverine are notably different from those of putrescine. Maximum potentiation is seen on simultaneous administration of cadaverine and histamine. The 40-min interval between doses reported by Parrot & Nicot (1966) to be required for potentiation with putrescine resulted in a decrease in effect with cadaverine. Thus, in one experiment, a 40-min interval between dosing with 100 mg cadaverine/kg and administration of 150 mg histamine/kg resulted in the death of only 60% compared with nearly 90% after simultaneous administration of these doses.

In a separate experiment, a comparison was made of the effects of simultaneous administration of histamine with putrescine or cadaverine. Animals were treated with 50 mg histamine/kg and either putrescine or cadaverine in a dose of 150 mg/kg. Although with the lag period this combination of histamine and putrescine doses would have been expected to kill all the animals (Parrot & Nicot, 1966), no deaths were observed after simultaneous administration. In contrast, one third of the animals died in the cadaverine–histamine test group. Finally, at the histamine levels used, potentiation was observed with cadaverine–histamine ratios of less than one. This is in contrast to the 5:1 putrescine–histamine ratio used in the putrescine potentiation studies (Parrot & Nicot, 1966).

The results are consistent with the hypothesis that histamine poisoning from fish products could be due to the potentiating effects of cadaverine. Fish poisoning apparently results from simultaneous consumption of histamine and its potentiator. Prior consumption of substances such as saponin and ethanol, which injure the intestinal mucosa and thereby increase the

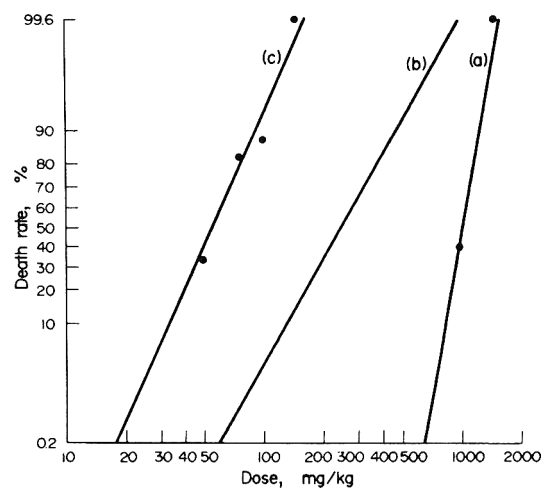

Fig. 1. Peroral toxicity curves for the dihydrochlorides of cadaverine (a), histamine (b) and various doses of cadaverine combined with 150 mg histamine/kg (c) in the guinea-pig. The ordinate (death rate) is plotted on a probability scale, and the abscissa (dose) on a logarithmic scale. The histamine curve was obtained from Parrot & Nicot (1966).

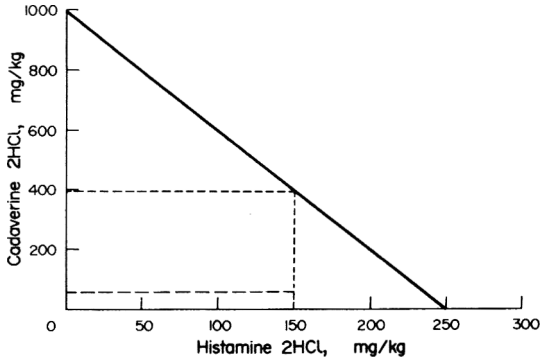

Fig. 2. Combinations of cadaverine dihydrochloride and histamine dihydrochloride doses (oblique line) that would result in the deaths of 50% of the guinea-pigs if the toxicities were additive, histamine and cadaverine by themselves having LD_{50} values of about 250 and 1000 mg/kg, respectively. For additive toxicities, a 50% death rate would result from histamine and cadaverine doses of 150 and 400 mg/kg, respectively (---), but a cadaverine dose of only 50 mg/kg is required (——), indicating potentiation of toxicity.

rate of histamine absorption, has been suggested as a factor in human poisoning (Geiger, 1955), but because of the relatively large numbers of individuals affected and the diverse circumstances under which poisonings have occurred, it appears unlikely that such substances play a general role in human poisoning from fish.

The relative levels of cadaverine and histamine present in toxic fish are of the same order of magnitude as those that show potentiation of histamine toxicity in the guinea-pig. Thus, levels of histamine exceed cadaverine levels in toxic and decomposed fish by a factor of approximately ten (Kim, 1978; Mietz, 1977). Lethal effects of histamine are potentiated in the guinea-pig by cadaverine levels as low as one fifth those of histamine. In contrast, the putrescine–histamine weight ratios in toxic fish (about 1:100) are very small compared to the relative levels (5:1) known to potentiate toxicity.

The possibility that toxic-fish components other than or in addition to cadaverine may be involved in poisoning cannot be ruled out on the basis of our results. Also, the question of the direct applicability to man of results obtained in the guinea-pig is unresolved. Nevertheless, the properties of cadaverine observed here suggest that it could be of importance, along with histamine, in the aetiology of scombroid fish poisoning.

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STUDIES ON THE ABSORPTION AND DISPOSITION OF ^{3}H-LABELLED TALC IN THE RAT, MOUSE, GUINEA-PIG AND RABBIT

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Abstract—The absorption and disposition of talc was studied in rats, mice and guinea-pigs, following a single oral dose of ^{3}H-labelled material. The translocation of talc in the rabbit urogenital tract was studied after single and multiple intravaginal applications. More than 95% of the dose of orally administered talc was excreted in the faeces within 3–4 days by all three species studied. Traces of radioactivity found in the urine probably reflected contamination of the samples by contact with the faeces and the presence of labile ^{3}H associated with the ^{3}H-labelled talc. No translocation of talc to the liver or kidneys was found. In the rabbit study, no translocation of talc into the ovaries was detected.

INTRODUCTION

Talc is a naturally occurring hydrous magnesium silicate with a general formula approximating to $Mg_3Si_4O_{10}(OH)_2$. It is used extensively in industry as well as having wide acceptance as a cosmetic and toiletry product. Although chemically inert, talc has been implicated in the development of tissue granulomas (Henderson, Melville-Jones, Barr & Griffiths, 1975; Migaki & Garner, 1969; Min, Gyorkey & Cain, 1974; Tye, Hashimoto & Fox, 1966), and a form of pneumoconiosis after inhalation (Miller, Tierstein, Bader, Bader & Selikoff, 1971). A report by Henderson, Joslin, Turnbull & Griffith (1971) indicated an association between cancer of the cervix and ovaries in humans and talc particles at these sites. The suggestion that there may be a link between the presence of talc particles and cancer was supported by studies on the tissues of Japanese patients with gastric cancer (Henderson, Evans, Davies & Griffiths, 1975; Matsudo, Hodgkin & Tanaka, 1974). Although the evidence presented in these studies is equivocal, the possibility of a causal relationship between particular types of tumours and the presence of talc has caused disquiet about its safety-in-use.

The development of a method for preparing ^{3}H-labelled talc (Gangolli, Crampton & Lloyd, 1973) facilitated the investigation of the biological fate of this talc preparation. In this paper, we present findings on the disposition of ^{3}H-labelled talc administered orally to rats, mice and guinea-pigs, and on the migration of talc into the urogenital systems of female rabbits.

EXPERIMENTAL

Materials. Talc (Purified, BP), supplied by Evans Medical Ltd, Liverpool, was labelled with ^{3}H as previously described (Gangolli *et al.*, 1973), using ^3H_2O with a specific activity of 500 mCi/ml. The specific activity of the talc preparation, determined by the method described below, was 100 μCi/g. The preparation conformed with the general physical characteristics of talc demonstrated by electron-microscopic and X-ray diffraction studies (J. K. Foreman, personal communication, 1972). In all the experiments, the ^{3}[H] talc preparation was administered as a suspension in aqueous glycerol jelly solution (10 mg/ml, 1 μCi/ml). This medium maintains a stable suspension of the talc particles for dosing, but rapidly dissolves *in vivo*.

Animals and diets. Female guinea-pigs of the Dunkin/Hartley strain (250–300 g body weight), male Wistar albino rats (120–150 g body weight), female mice of the LACA strain (30–40 g body weight) and female Large White rabbits (3–4 kg body weight) were used in these studies.

The rats and mice were maintained on Spillers Laboratory Small Animal Diet No. 1, and the guinea-pigs and rabbits on Oxoid Diet SG1. All animals were given both food and water *ad lib.* and kept at 20 ± 2°C. The guinea-pigs were given vitamin C supplement in the drinking-water. The rats, mice and guinea-pigs were housed individually in all-glass metabolism cages (Jencon Scientific Ltd., Hemel Hempstead, Herts). The rabbits in aluminium metabolism cages (All-Type Tools Ltd., Woolwich, London), equipped with a grid floor to retain faeces and having an inverted cone-shaped tray beneath with a hole in the apex to allow the urine to run into a collection flask.

Stability of the ^{3}H-labelled talc. ^{3}[H] Talc (30 mg, 0·3 μCi) was boiled in water (10 ml) for 30 min. The suspension was cooled and filtered through a 45-μm millipore filter and the radioactivity in the filtrate was determined. In addition two male rats were injected sc with ^{3}H-labelled talc suspension (0·1 ml, 10 mg). After 5 days, the granuloma produced at the injection site was excised and the total radioactivity was determined.

Biological disposition studies—oral administration. Three male rats were each given a single dose of ^{3}H-labelled talc by oral intubation (0·5 ml suspen-

sion/100 g body weight) providing a dose level of 50 mg talc/kg body weight. Urine and faeces were collected 24-hr intervals for 4 days and on day 10. At the end of the experimental period the liver, kidneys and gastro-intestinal tract were removed, samples of urine (0·5 ml) were assayed directly for radioactivity and the radioactivity in the faeces and tissues was determined by combustion. Three further rats were given orally six daily doses of ^{3}[H]talc (50 mg talc/kg body weight/day), and killed 10 days after the last dose. Radioactivity in excreta and tissues was determined as described for the single-dose experiment. Each of three female guinea-pigs was given a single oral dose of ^{3}H-labelled talc at a level of 25 mg/kg. Urine and faeces were collected at 24-hr intervals as in the previous experiment. After 10 days the animals were killed and liver, kidneys and gut were removed. Radioactivity in the samples was determined as described for the rat. In addition four female mice were given a single oral dose of ^{3}H-labelled talc at a level of 40 mg/kg. Two animals were killed at 6 hr and two at 24 hr. Urine and faeces were collected and the complete gastro-intestinal tract was separated from the carcasses. Radioactivity was determined in the urine, in the combined faeces and large intestine, in the combined stomach and small intestine and in the carcass.

Biological disposition studies—intravaginal administration. Three rabbits were given a single intravaginal dose of ^{3}H-labelled talc suspension (0·5 ml) and placed in metabolism cages. Urine was collected at 24-hr intervals for 3 days. The rabbits were then killed and the urogenital tract was dissected out. The total ratioactivity was determined in the urine, the ovaries, the fallopian/uterine tubes and cervix, and the vagina and bladder. A further three rabbits were given six daily doses of ^{3}H-labelled talc intravaginally, and then killed 72 hr after the final dose. The urogenital tracts were dissected out and the radioactivity was determined as in the single-dose experiment.

Radioactivity determinations. Radioactivity was measured in a Packard 2650 liquid scintillation counter and efficiency was determined by the external channels-ratio method. Urine (0·5 ml) was counted in a scintillation fluid of toluene–2-ethoxyethanol (1:1, v/v) containing 2,5-diphenyloxazole (PPO, 0·4%, v/v). Faeces, tissues and portions of talc suspension were oxidized in a Packard 306 Sample Oxidiser and the ^3H_2O produced was counted in 15 ml Monophase 40 (Packard Instruments, Des Plaines, IL, USA). Tritium recovery was 97–99%.

RESULTS

Both *in vitro* and *in vivo* levels of exchange with external hydrogen atoms were determined for the samples of ^{3}H-labelled talc used in these experiments. Less than 0·9% of the label was lost after 30 min in boiling water, and *in vivo* 97·3–99·4% of the label was recovered in the sc granulomas excised from rats 5 days after talc injections. No radioactivity was detected in the urine of these rats.

Following the oral administration of a single dose of ^{3}H talc to rats, approximately 75% of the label was recovered in the faeces within 24 hr (Table 1). Most of the radioactivity (95·8%) had been excreted after 96 hr, and only a trace of radioactivity (3200 ± 340 dpm, equivalent to 0·08% of the administered dose) remained in the gastro-intestinal tract at 240 hr. Less than 2% of the administered radioactivity was accounted for in the urine during the course of the experiment. However, there was no radioactivity in the liver or kidneys after 10 days.

In the case of rats given six daily doses of ^{3}[H]talc orally, there was no radioactivity in the day-10 faeces or in the livers of the animals at this time, although there was a trace of activity in the kidneys (less than 0·02% of the total dose).

After administration of a single oral dose of ^{3}[H]talc to guinea-pigs, almost all of the radioactivity was recovered in the faeces by 96 hr, although the activity recovered in the 24-hr faeces was substantially lower than from the rat (Table 2). Very little radioactivity appeared in the urine (less than 0·2% of the dose), and although the radioactivity remaining in the gastro-intestinal tract at 96 hr was higher than in the rat, by 10 days only a trace of activity (less than 0·03% of the dose) remained.

Following oral administration of [^{3}H]talc to mice, all of the radioactivity was found in the gastro-intestinal tract and faeces at 6 and 24 hr. No radioactivity was detected in the rest of the carcass (Table 3).

The administration of a single dose of ^{3}H-labelled talc into the rabbit vagina resulted in radioactivity in the urogenital tract being detected only at the site of adminstration after 72 hr (0·004 ± 0·001% of dose). The sensitivity of the method is such that 0·25 μg of talc could be detected. No attempt was made to quantitate the total recovery of radioactivity. Seventy-two hours after the last of six daily intravaginal doses, radioactivity was detected in the urogenital tract at the site of administration (0·035 ± 0·024% of dose)

Table 1. *Excretion of ^{3}H-labelled talc following oral administration to rats*

Time (hr)	Percentage of dose excreted* in		
	Faeces	Urine	Total
0–24	74·8 ± 8·9	1·05 ± 0·51	75·9 ± 9·1
24–48	9·5 ± 8·5	0·58 ± 0·26	10·1 ± 8·7
48–72	10·8 ± 17·2	0·06 ± 0·08	10·9 ± 17·1
72–96	0·6 ± 0·2	<0·01	0·6 ± 0·2
216–240	0·07 ± 0·02	<0·01	0·07 ± 0·02
0–96	95·8 ± 9·4	1·67 ± 0·30	97·5 ± 10·3

*Expressed as mean ± SD for three animals.

Table 2. *Excretion of ^3H-labelled talc following oral administration to guinea-pigs*

Time (hr)	Percentage of dose excreted* in		
	Faeces	Urine	Total
0–24	31·4 ± 14·4	0·2 ± 0·01	31·6 ± 14·4
24–48	39·3 ± 6·5	<0·01	39·3 ± 6·5
48–72	18·9 ± 15·8	<0·01	18·9 ± 15·8
72–96	4·8 ± 4·5	<0·01	4·8 ± 4·5
216–240	0·03 ± 0·01	<0·01	0·03 ± 0·01
0–96	94·4 ± 6·2	0·2 ± 0·01	94·6 ± 6·3

*Expressed as mean ± SD for three animals.

Table 3. *Distribution of ^3H in mice following oral administration of ^3H-labelled talc*

Time (hr)	Percentage of dose recovered* in				Total recovery (% of dose)
	Urine	Large intestine and faeces	Small intestine and stomach	Carcass	
0–6	0·66	94·8	9·0	<0·005	104·5
	0·0	96·2	7·1	<0·005	103·3
0–24	1·26	98·9	4·4	<0·005	104·6
	1·49	101·1	6·4	<0·005	109·0

*Values represent results from individual mice.

and a small amount was found associated with the cervix and fallopian/uterine tubes (0·006 ± 0·003% of dose). However, no radioactivity was found in the ovaries.

DISCUSSION

The widespread use of talc in the food industry and the consequent exposure of the general population to this material has led to concern over the possible involvement of talc in the aetiology of stomach cancer and tissue-granuloma formation. The present study has demonstrated that orally administered talc is not translocated from the gastro-intestinal tract of rats, mice or guinea-pigs, but that it is almost entirely excreted in the faeces over a period of 3–4 days. Following a single dose of ^3H-labelled talc to rat and guinea-pigs, less than 0·08 and 0·03%, respectively, of administered radioactivity remains associated with the gastro-intestinal tract after 10 days. The low level of radioactivity in the urine probably represents contamination of the samples with faeces during the separation of the excreta in the metabolism cages, and labile ^3H associated with the ^3H-labelled talc samples. However labile ^3H has been shown to represent less than 1% of the administered radioactivity.

The recent finding that ovarian tumours may contain talc particles (Henderson *et al.* 1971) has led to concern over the application of talc to the female urogenital region both as a cosmetic preparation and in association with gynaecological examination. Our study shows that talc does not migrate to the ovaries of rabbits given the material intravaginally after either single or repeated applications. Our finding that talc is not translocated from the site of administration is supported by a recent study by Wehner, Wilkerson, Cannon, Buschbom & Tanner (1977), who found that talc administered by inhalation to hamsters was not translocated from the respiratory tract, and there was no evidence of talc in the liver, kidneys or ovaries of the animals. Trace amounts of radionuclides in the urine of exposed animals were possibly due to leaching from the talc.

The results of this study suggest, therefore, that talc administered orally to rats, guinea-pigs and mice remains within the gastro-intestinal tract and is completely eliminated within 3–4 days. The study also shows that talc applied intravaginally to the rabbit does not migrate to the ovaries.

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Review Section

PROBLEMS INVOLVED IN AND A COMPARISON OF METHODS FOR THE DETERMINATION OF TOTAL MIGRATION FROM PACKAGING MATERIALS INTO FATTY FOODS*

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Summary—All procedures currently used to evaluate the total migration of components from packaging materials into fat-releasing foodstuffs are based on the determination of the weight of a sample of the packaging material before and after its storage in a test fat under standard conditions, a correction being applied for the residual fat retained in/on the sample. The latter may be determined by physical or chemical methods, the calculation being based in most cases on a single component of the triglyceride mixture that has migrated into the sample. With most test fats, differential migration of the individual components of the fat into and incomplete extraction of the absorbed fat from the test polymer are serious sources of error. Fewer problems are associated with the use of ^{14}C-labelled HB 307 as the fat simulant and this method has now been developed to a high degree of accuracy for use with all types of plastics, including the highly cross-linked and insoluble types. Following a description of the revised method and its development, the additional difficulties encountered in the determination of total migration from cardboard/plastics composites are discussed.

Introduction

The decision on whether a packaging material complies with the requirements of the Food Law is closely connected with the question of the extent to which the components of that packaging material will migrate into the packed food. In order to answer this question, so-called migration tests are carried out under conditions representative of those prevailing in practice, using test systems comprising the packaging material and either foods or appropriate food simulants.

A distinction is made between total migration and specific migration, the latter being the transfer of a defined, toxicologically interesting component of the packaging material, such as a plastics additive or a monomer which is to be determined quantitatively, while in the former case the sum of all migrating components is considered.

The report is concerned exclusively with the determination of the total components migrating from packaging materials into fatty foodstuffs (the 'total migrate') with special reference to the difficulties and sources of error involved in the practical performance of such determinations.

Generally accepted principle for the determination of total migrate

The literature of the last 20 years has described several basically different procedures for the direct and indirect determination of total migrates into test fats. When these procedures were checked under practical conditions, it became apparent that only the approach proposed by the Organisation der Kunststoff-Hersteller der EWG (BITMP, 1972) for the determination of total migrate (Fig. 1) met all the requirements adequately. Consequently, this method has been generally accepted in principle. It involves storage of a sample of the packaging material, of known weight (Gv) and surface area, in the test fat under prescribed experimental conditions, after which the sample is freed from externally adhering fat and reweighed (weight Gn). Subsequently, the amount of fat retained on or in the sample (Fp) is determined quantitatively by a physical or chemical method, and the total migrate (GM) is calculated according to the equation: $GM = Gv - (Gn - Fp)$.

Differences in procedures for the determination of total migrate

All the methods published during recent years and now used for the determination of total migration into fat-releasing foodstuffs are based on the principle described above. However, they differ considerably in the type of test fat used and in the analysis of the amount of fat (Fp) absorbed by the sample of packaging material during contact with the test fat.

Thus, in some working methods, unsaturated edible fats, such as olive oil and sunflower oil, are specified as contact media. In these cases, Fp is calculated from the determination of the iodine value (Pallière, 1972) or of the oleic or linoleic acid present, these acids being determined by gas chromatography in the form of their methyl or silyl esters (Ostromow & Canji, 1973; Piacentini, 1972; Rossi, Sampaolo & Gramic-

*Based on a paper presented at the Second International Symposium on Migration held on 3 and 4 November 1976 in Hamburg.

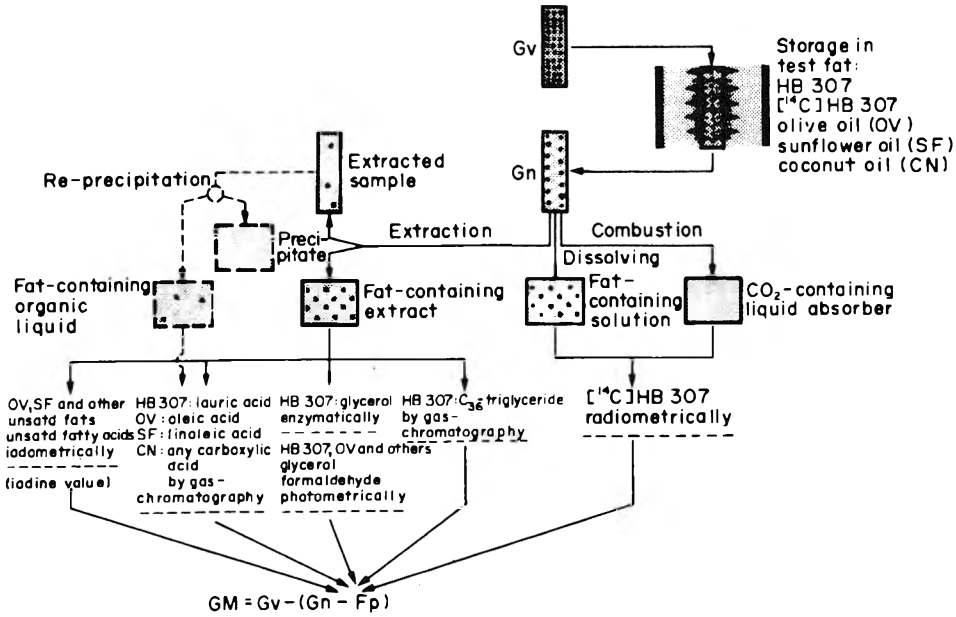

Fig. 1. Sequence of procedures used for the determination of total migration of packaging-material components into unlabelled or ^{14}C-labelled fat simulant HB 307 or unlabelled natural oils.

cioni, 1972; Sampaolo, Gramiccioni, Rossi, Esposito & Binetti, 1972). The unsaturated fatty acids present in high concentration in the test fats are thus used for the determination of Fp.

Coconut oil has also been used as a migration medium (Piacentini, 1972). In this case, the retained fat (Fp) is again determined by gas-chromatography of a selected carboxylic acid methyl or silyl ester, the detection of which is not sensitive to interference by extractable components of the packaging material.

Apart from these edible oils, the main fat simulant used for the determination of total migrates has been the synthetic triglyceride mixture HB 307 in inactive (Figge, Eder & Piater, 1972) or ^{14}C-labelled form (Figge, 1973a & 1976). Among others, the use of this simulant offers the advantage that it permits simultaneous and sensitive determination of the specific migration of single components of the packaging material, such as individual plastics additives or monomers in the same batch (Koch, 1972 & 1974). Consequently several working methods elaborated in recent years are based on the use of inactive or ^{14}C-labelled HB 307 (Bundesgesundheitsamt, 1975; Figge, 1973a,b & 1975; Koch & Kröhn, 1975; Pfab, 1972; van Battum & Rijk, 1972). These methods differ only in the analytical procedure used to determine the amount of retained fat (Fp). When the unlabelled fat simulant is used, the procedure may involve either the gas-chromatographic determination of the lauric acid in the form of its methyl ester (van Battum & Rijk, 1972), lauric acid being present in the test fat at levels up to *c.* 50%, the photometric (Pfab, 1972) or enzymatic (Koch & Kröhn, 1975) determination of the glycerol content, or the direct gas-chromatographic determination the triglyceride mixture via the C_{36} component (Koch & Kröhn, 1975). In the ^{14}C-labelled HB 307, the radiocarbon is evenly distributed among all fatty acids and the glycerol, so if [^{14}C]HB 307 is used as the simulant, the amount of fat (Fp) absorbed by the packaging material sample can be detected easily and precisely by a determination of the radioactivity (Bundesgesundheitsamt, 1975; Figge, 1973a,b & 1975).

Difficulties and sources of error in the practical performance of total-migrate determinations

The use of these working methods will lead to realistic and legally binding values for the total migrate only if the weights of the packaging-material samples before and after storage in the test fat, as well as the amount of retained fat, are determined with high accuracy. The determination of Gv and Gn for plastics can be carried out simply and sufficiently accurately provided both weighings are done under identical atmospheric conditions. In contrast, the determination of Fp requires the utmost attention. Except for the [^{14}C]HB 307 method, a common feature of all the procedures for determining total migrate is the use of the penetration of a single component of the triglyceride mixture into the plastics sample as the basis for the determination of Fp. It is presumed that all components of the test fat, irrespective of their molecular weight, polarity and degree of saturation, will penetrate into the plastics to the same extent in proportion to their original concentration. Moreover, it is taken for granted that all components of the test fat that have migrated into the plastics sample can be re-extracted quantitatively with the usual solvents or extractants, such as *n*-pentane, chloroform, Frigen 113 and others. As discussed below, however, experiments have shown that these basic assumptions are not correct for many plastics/test-fat systems. Moreover, when total migration from cardboard/plastics laminates is under investigation, the determination of Gv and Gn presents particular difficulties.

Changes in the carboxylic acid composition of test fats during migration into plastics

The first confirmation that the composition of a test fat may change during its penetration into plastics was obtained when Koch & Figge (1975) determined the total migrations from high-density polyethylene (HD-PE films, 100 μm thick), low-density polyethylene (LD-PE, 100 μm thick), plasticized PVC (200 μm thick), rigid PVC (200 μm thick), plasticized polyvinylidene chloride (100 μm thick) and rubber (500 μm thick) into sunflower oil and the triglyceride mixture HB 307. In these tests, the methyl linoleate and laurate peaks of the gas chromatograms were evaluated to determine Fp and, in addition, the peak area distribution of the fatty acid methyl esters in the interesterification products of the test oils were compared before and after migration into the plastics. The storage conditions used for these studies and the results of the gas-chromatographic investigations are given in Tables 1 and 2.

Table 1 shows how the ratio of oleic to linoleic acid in sunflower oil changed during penetration into plasticized PVC. In the original oil the ratio was 1:1·98, while in the extract of the film it was 1:1·64. Thus, the relative increase in oleic acid was 12% and the decrease in linoleic acid 6%. Even more marked was the selectivity of HD-PE towards the two main components of the sunflower oil (Table 1), with a relative increase in oleic acid and decrease in linoleic acid of more than 20%. Moreover, an increase in saturated carboxylic acids became more marked with decreasing chain length.

In contrast, the shifts in the composition of sunflower oil on penetration into LD-PE and rigid PVC are only low (Table 1), but in spite of this, the error in the determination of Fp for the PVC amounted to about 7% when the peak area of the linoleic acid methyl ester was evaluated and to about 14% for the oleic acid methyl ester peak. Because of the low level of absorption of oil by rigid PVC, however, the absolute error in the determination of the total migrate would be insignificant. Particularly wide shifts in the carboxylic acid composition with a simultaneously strong absorption of fat were observed after a 10-day contact of rubber with sunflower oil and, in contrast to the examples already discussed, the oleic acid content decreased in this case by about 30% and the linoleic acid content increased by about 17%.

When the test fat HB 307 penetrated into plastics (Table 2), there was again a shift in the proportions of triglycerides in relation to the number of carbon atoms, but in all the plastics/test-fat systems investigated so far, the concentration of lauric acid, which is decisive for the determination of the total migrate, has been found to remain nearly constant, whereas that of the shorter-chain carboxylic acids increases and that of the longer ones decreases.

Table 1. *Changes in the carboxylic acid composition of sunflower oil on penetration into plastics films held in single-sided contact with the oil for 10 days at 40 C*

Plastics material	Original oil or plastics extract	Concn ratio (peak area %)* of carboxylic acids				
		14:0	16:0	18:0	18:1	18:2
Plasticized PVC	Oil	—	—	—	33·52	66·48
	Extract	—	—	—	37·89	62·11
HD-PE	Oil	0·08	7·81	4.08	28·01	60·02
	Extract	0·78	12·15	6·50	34·33	46·24
LD-PE	Oil	0·31	7·60	3·66	31·75	56·68
	Extract	0·64	9·63	4·76	31·82	53·15
Rigid PVC	Oil	—	—	—	35·27	64·73
	Extract	—	—	—	40·50	59·50
Rubber	Oil	—	—	—	35·90	64·10
	Extract	—	—	—	24·98	75·02

PVC = Polyvinyl chloride HD-PE and LD-PE = High- and low-density polyethylenes
*Corrected peak areas of methyl esters of the carboxylic acid/test film = 100%. Values are means for two determinations.

Table 2. *Changes in the carboxylic acid composition of the synthetic triglyceride HB 307 on penetration into plastics films held in single-sided contact with the oil for 10 days at 40 C*

Plastics material	Original fat† or plastics extract	Concn ratio (peak area %)* of carboxylic acids						
		6:0	8:0	10:0	12:0	14:0	16:0	18:0
Rubber	HB 307	0·05	4·60	9·04	52·25	14·73	9·18	10·15
	Extract	0·79	10·32	12·01	51·95	12·65	6·51	5·77
Plasticized PVDC I	HB 307	0·1	4·2	7·2	56·1	14·5	8·2	9·7
	Extract	0·7	8·8	9·9	54·1	12·0	6·6	7·9

PVDC = Polyvinylidene chloride
*Corrected peak areas of methyl esters of carboxylic acid/test film = 100%. Values are means of two determinations.
†The HB 307-batches differed in their fatty acid compositions from the commercial test fat.

Shifts in the triglyceride composition of the test fat HB 307 during its penetration into plastics

An immediate picture of the selection process occurring in the polymer can be obtained by evaluation of the gas chromatograms of the intact triglyceride mixtures before and after their migration into the plastics.

Table 3 shows a shift in the concentration of the triglycerides towards shorter chain lengths in the retained fat in all cases investigated, and the extent and direction of the changes confirm the results already obtained with the carboxylic acids. The greatest effect was observed with plasticized polyvinylidene chloride (plasticized PVDC II). Here, the portion of triglycerides with less than 36 carbon atoms increased from 29·5 to 54·2 and that of the triglycerides with more than 36 decreased from 51·0 to 27·4%.

As with the C_{12}-carboxylic acids, the concentration of the C_{36}-triglycerides was practically constant in all cases.

Influence of the change in fat composition on the determination of total migrate

Some practical examples from our studies will demonstrate the absolute errors in the total-migrate determination that can result from this preferential absorption of certain fat components into the plastics/test-fat systems.

The total migrate from LD-PE stored in sunflower oil amounted to 8·4 mg/dm^2 when the amount of absorbed fat (Fp) was determined from the methyl linoleate peak of the gas chromatogram but to 21·9 mg/dm^2 when the methyl oleate peaks were evaluated (all values cited being means of three separate determinations). This discrepancy can be explained by the opposing changes in concentration of the two fatty acids during the penetration of sunflower oil into LD-PE. The correct value calculated on the 'true' linoleic acid content of the fat in the plastics was 15·3 mg/dm^2.

Determination of the total migrate from rubber into sunflower oil yielded values of 93·4 or −9·1 mg/dm^2, according to whether the methyl linoleate or methyl oleate peak was used for the determination of Fp. In comparative migration tests with the same rubber film in the test fat HB 307, determination of Fp by evaluation of the methyl laurate peak gave a value of 43·9 mg/dm^2. If we consider the reduction in lauric acid content from 52·25 to 51·95% which occurs when HB 307 penetrates into rubber, the 'true' migrate amounts to 48·7 mg/dm^2. Thus, using the lauric acid method, the error amounts to 4·8 mg/dm^2.

Naturally, the enrichment of the short-chain triglycerides in the plastics also affects the glycerol methods for the determination of total migrate, because of the increase in glycerol content resulting from this enrichment. In Table 4 the glycerol contents calculated from the triglyceride distribution of the absorbed fats are compared with those of the original HB 307 test fats.

Table 3. *Changes in the triglyceride composition of HB 307 on penetration into plastics films held in single-sided contact with the fat for 10 days at 40 C*

Material analysed	Type of test film	Concn ratio (peak area %)* of triglycerides of carbon no.		
		24 + 2n (n = 0–5)	36	38 + 2n (n = 0–5)
HB 307†	—	29·5	19·5	51·0
Plastics extract	HD-PE	35·6	21·2	43·0
	Plasticized PVC	47·8	20·6	31·6
	Plasticized PVDC II	54·2	18·4	27·4
HB 307†	—	31·1	20·5	48·4
Plastics extract	LD-PE	33·0	20·7	46·3
HB 307†	—	35·07	20·53	44·40
Plastics extract	Rubber	45·37	20·51	34·05

HD-PE = High-density polyethylene PVC = Polyvinyl chloride PVDC = Polyvinylidene chloride
LD-PE = Low-density polyethylene
*Corrected peak areas of the triglycerides/test foil = 100%. Values are means of two determinations.
†HB 307 batches differed in their triglycerides composition from the commercial test fat.

Table 4. *Differences in the glycerol content of test fat HB 307 before and after penetration into different test films and errors resulting from these differences in total-migrate determinations by the glycerol method*

Type of test film	Glycerol content (%)			Amount (mg/dm^2) of fat extracted (Fp)*	Systematic error in total-migrate determination (mg/dm^2)
	HB 307 used	Film extracts	Relative deviation		
Rubber	14·35	14·78	3·7	288·9	8·7
Plasticized PVC	14·16	14·82	4·7	118·3	5·6
HD-PE	14·16	14·40	1·7	54·4	0·9
Plasticized PVDC II	14·16	15·11	6·7	6·3	0·4
Plasticized PVDC I	14·18	14·76	4·1	14·7	0·2

PVC = Polyvinyl chloride HD-PE = High-density polyethylene PVDC = Polyvinylidene chloride
*Determined by the lauric acid method.

In the different types of plastics material, they were between 1·7 and 6·7% higher than in the HB 307 batches used. Since the glycerol concentrations used in the determinations were thus too low, the Fp values obtained, and therefore the values of total migrate, were too high.

The errors given in the final column of Table 4 for the determination of total migrate by the glycerol method have been calculated from the difference in glycerol contents between the fat used and that extracted and from the absolute amount of fat extracted from the plastics sample. The greatest error, found with the rubber samples, amounts to 8·7 mg/dm^2 contact areas, and is thus higher than the error associated with the 'lauric acid' method. A fairly high error of 5·6 mg/dm^2 is also apparent with plasticized PVC, but for the remaining examples investigated, the calculated systematic errors lie below 1 mg/dm^2.

Residual levels of test-fat components in extracted plastics and rubber samples

An additional and largely undervalued source of error arises from the fact that even after the most intensive extraction, some of the absorbed fat may be retained in the sample. The level of residual fat in an extracted sample of packaging material may depend on the chemical and physico-chemical structure of the packaging material, on the type of test fat and on the swelling effect of the extractant.

The most important prerequisite for a rapid and complete extraction of the absorbed fat from samples of packaging material is fulfilled, if the texture of the packaging material is swollen as much as possible by an extractant that readily dissolves fats. Therefore, we checked numerous fat-dissolving organic liquids for their swelling efficiency towards types of plastics and rubber differing widely in chemical structure as well as in physico-chemical properties, such as degree of polymerization and cross-linking. In these investigations, which will be reported in detail elsewhere (K Figge, M. Scholle & J. Koch, unpublished data 1976), it was found that among other organic liquids, Frigen 113 (1,1,2-trichloro-1,2,2-trifluoroethane) was a suitable extractant for plastics, as was chloroform for rubber samples. Therefore, these two liquids were the principal ones used to check the extractability of the test-fat components that had penetrated into plastics and rubber samples.

The results of some of these extraction experiments are summarized in Tables 5 and 6. Plastics and rubber samples differing markedly in their chemical and physico-chemical properties were stored for 10 days or 24 hours at 40°C in [^{14}C]HB 307 or in ^{14}C-labelled olive oil (prepared by incorporating

Table 5. *Residual amounts of components of HB 307 or of olive oil in plastics samples in contact with the fat for 10 days at 40 C and subsequently extracted with Frigen 113* for 18 hours*

Plastics films				Residual content of components of			
				HB 307		Olive oil	
Type	Melt index† or designation	Thickness (μm)	No. of single determinations	mg/dm^2 KF‡	%§	mg/dm^2 KF‡	%§
Polyethylene	1	550	15	3·8 ± 11·8	0·79	0·2 ± 8·1	0·04
	7	550	15	1·5 ± 13·4	0·34	0·2 ± 6·0	0·05
	20	550	15	0·4 ± 28·2	0·12	0·2 ± 6·3	0·08
Polystyrene	3	1100	15	40·1 ± 6·7	9·60	37·2 ± 9·5	11·4
ABS‖	2·5	550	15	0·7 ± 19·6	0·15	0·8 ± 7·1	0·28
Polystyrene¶	846 LE	380	6	6·4 ± 4·6	0·32	11·4 ± 2·5	0·57
	476 LW	620	6	6·0 ± 19·6	0·21	1·3 ± 12·8	0·04
	143 E	80	6	0·6 ± 31·4	0·15	1·6 ± 31·1	0·30
	165 H	50	6	0·2 ± 17·8	0·04	0·3 ± 26·1	0·06
	168 N	60	6	0·1 ± 27·0	0·03	0·1 ± 13·1	0·02
Rigid PVC**	Solvic 229††	470	15	ND	—	0·2 ± 14·3	0·007
HD-PE**	Lupolen 5261‡‡	470	15	ND	—	2·1 ± 1·7	0·088
LD-PE**	Lupolen 1800‡‡	450	15	ND	—	0·3 ± 7·8	0·016
Polypropylene**	Propathene LYH-42§§	460	15	0·3 ± 30·2	0·013	0·6 ± 3·9	0·025
HI-PS**	466 I‡‡	500	15	ND	—	0·3 ± 8·6	0·011
Polystyrene**	3187, instab.‡‡	580	15	0·2 ± 16·3	0·008	0·2 ± 10·3	0·006
Polyamide**	Ultramid B3‡‡	450	15	ND	—	1·5 ± 4·5	0·058
Polycarbonate**	Unknown	500	15	ND	—	ND	—

KF = Contact area between sample and test fat ABS = Acrylonitrile-butadiene-styrene terpolymer
HD-PE and LD-PE = High- and low-density polyethylenes HI-PS = High-impact polystyrene

*1,1,2-Trichloro-1,2,2-trifluoroethane.

†See standards ASTM D 1238 (USA), DIN 53735 (BRD) and ISO R 292.

‡Values are means ± standard deviations in %, for the stated no. of determinations (except for the italicized figures, which are based on 12 determinations).

§Percentage of the sum of the mean amounts of fat in the extracts and extracted samples.

‖Films used in a collaborative test organized by the EEC Committee.

¶Films kindly provided by BASF AG, Ludwigshafen, without further information.

**Films prepared by Unilever Forschungsgesellschaft mbH, Hamburg.

††Manufactured by Solvay Werke, Düsseldorf.

‡‡Manufactured by BASF AG, Ludwigshafen.

§§Prepared by Unilever Forschungegesellschaft mbH.

[1-^{14}C]oleic acid into refined olive oil). Subsequently, they were freed from externally adhering test fat in the way prescribed in the working methods for the determination of total migrate. After this treatment, the plastics samples were extracted with Frigen 113 for 18 hours and the rubber samples with chloroform for 24 hours using a Soxhlet apparatus, and the residues of test-fat components in the extracted samples were determined by radioanalysis.

The first demonstration that plastics are capable of retaining absorbed test-fat components even when extractants of apparently optimal efficiency are used was obtained by radioanalysis of different plastics films which had been stored in ^{14}C-labelled HB 307 or olive oil and then extracted (Table 5). These films originated from a collaborative test arranged by the EEC Committee. The high content of residual test-fat components in polystyrene was striking: in the case of the olive oil it amounted to 37 mg/dm^2 and with the simulant HB 307 it was 40 mg/dm^2 film area.

In view of this finding, five further types of polystyrene were investigated in the same way for their capacity to retain test-fat components. Particularly high residue levels were found in the polystyrene films 846 LE and 476 LW, which were 380 and 620 μm thick respectively (Table 5). In contrast, films 165 H (50 μm thick) and 168 N (60 μm) showed a distinctly lower retention capacity towards test-fat components under the same extraction conditions.

Low levels of residual test-fat components were found also after the extraction of a series of plastics films produced in our pilot plant from weakly pre-stabilized basic polymers (Table 5). Strikingly high levels of test-fat residues were found in commercial rubbers after extraction for 24 hours with chloroform (Table 6). Following exposure to HB 307 they lay between 17 and 33 mg/dm^2, while with olive oil they were between 18 and 71 mg/dm^2 film surface. Attempts to reduce these levels by a 24-hour 'hot' extraction with chloroform were unsuccessful. The examples given in Table 6 show that even after this intensive extraction the residual amounts of test-fat components retained by the rubbers were still too high to be acceptable for conventional methods for determining total migrate.

Because of all these difficulties and sources of error in the practical performance of total-migrate determinations, we take the view that, particularly in doubtful cases, only the [^{14}C]HB 307 method will yield safe and realistic total-migrate values, and this will depend on the amount of retained fat (Fp) being determined by the procedure presented schematically in Fig. 2. In the simplest procedure for the determination of Fp in plastics or rubber samples that have been stored in [^{14}C]HB 307, the sample is dissolved in an appropriate solvent and the radioactivities of aliquots of this solution are determined in a β-scintillation counter using a suitable scintillator liquid. The radioactivity of the total solution is then directly proportional to the amount of fat retained in the sample. However, this procedure can be applied in only a few cases since many types of plastics and rubber are insoluble.

On the other hand, Fp can be determined in any plastics or rubber sample by cutting the sample into pieces of about 200 mg or pulverizing it, while cooled with liquid nitrogen, in a steel vessel containing steel balls, subjecting 5–10 random samples of the cut or pulverized specimen to complete combustion to form carbon dioxide, absorbing this quantitatively in 2–3 ml of an appropriate organic base, taking the CO_2-containing absorbent quantitatively into 18 ml scintillator liquid and measuring in a β-scintillation counter. From the mean specific radioactivity of the random samples and the sample weight (Gn) the total radioactivity of the plastics or rubber sample is calculated, and this is directly proportional to the amount of fat retained in the sample.

Table 6. *Residual amounts of components of HB 307 or of olive oil in rubber samples in contact with the fat for 24 hours at 40 C and subsequently extracted for 24 hours in a standard or 'hot-extraction' Soxhlet apparatus*

Rubber films*		Residual content of components of			
		HB 307		Olive oil	
Designation	Thickness (μm)	mg/dm^2 KF†	%‡	mg/dm^2 KF†	%‡
Standard Soxhlet extraction					
NA 467 1 light	2020	26·4 ± 11·6	0·15	19·0 ± 15·8	0·11
A 145/1 red	2500	23·1 ± 8·8	0·08	71·0 ± 7·7	0·26
NL 280/1 black	2250	29·8 ± 20·9	0·09	64·4 ± 7·5	0·22
C 21	2780	17·4 ± 22·7	0·10	21·3 ± 8·2	0·11
C 22	2340	33·0 ± 10·4	0·17	18·7 ± 19·4	0·10
C 33	2130	30·5 ± 14·7	0·13	32·1 ± 4·0	0·14
'Hot' Soxhlet extraction					
NA 467/1 light	2020	41·9 ± 9·1	0·23	29·0 ± 5·9	0·16
C 21	2780	36·7 ± 9·3	0·12	66·8 ± 3·8	0·22

KF = Contact area between sample and test fat

*Kindly provided by Continental Gummiwerke AG, Korbach, and Giessener Gummiwarenfabrik, Poppe a. Co., without further information. Fat absorption, determined gravimetrically, varied from 193 to 3754 mg/dm^2 according to the sample.

†Values are means ± SD in % for nine separate determinations.

‡Percentage of the sum of the mean amounts of fat in the extracts and extracted samples.

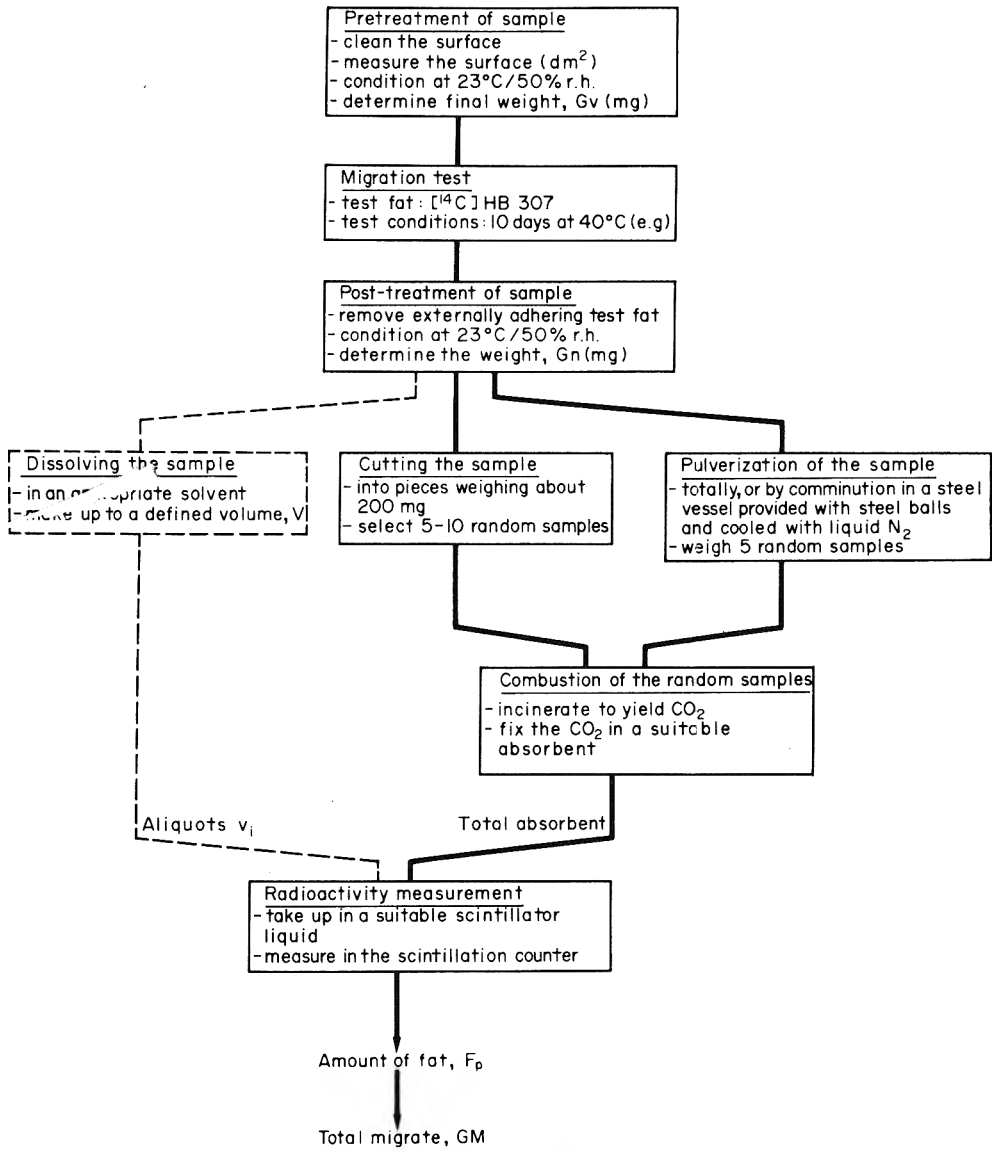

Fig. 2. Schematic representation of the procedures involved in the determination of the amount of fat (Fp) present in packaging-material samples stored in contact with the test fat [^{14}C]HB 307.

Difficulties in the practical performance of total-migrate determinations on cardboard/plastics laminates

The methods for the determination of the total components migrating from plastics into fat-releasing foodstuffs cannot be applied readily to cardboard/plastics laminates. In addition to the problem involved in the determination of the amount of fat penetrating into the packaging material, it is difficult with the laminates to weigh the samples exactly before (Gv) and after (Gn) contact with the test fat, because the weight of a cellulose-containing material, such as is present in a cardboard/plastics laminate, depends to a great extent on the atmospheric conditions to which it has been exposed previously and on the conditions prevailing at the moment of weighing.

To investigate this dependency, a series of 70 cardboard samples of the same origin was divided into seven groups, which were, in sequence, air-conditioned for 24 hours at 20°C and 65% relative humidity, weighed, subjected to widely differing atmospheric conditions, air-conditioned again at 20°C/65% relative humidity for 24 hours and reweighed. It is clear from Table 7 that for each group the weight of the stored and re-acclimatized board was distinctly higher than the initial weight, the greatest deviation from the initial weight being an increase of some 2% (sample group 2). From these results, it is evident that a 24-hour period of air-conditioning is quite inadequate for the determination of the weights (Gv and Gn) of a sample of cardboard/plastics laminate. On the contrary, it is necessary to expose the sample to an appropriate atmosphere until it shows a constant weight. In practice this means that the difference between two weights of the same sample obtained at an interval of 24 hours must be smaller than 1 mg.

In a further experiment we intended, first, to establish whether a cardboard sample, which has been air-conditioned to constant weight and then dried to con-

Table 7. *Initial and final weights of cardboard samples subjected to different temperature and humidity conditions during an intermediate storage*

Group no.	Mean weight* (mg)		Difference (mg) between initial and final weights	
	Initial†	Final†	Mean	Range
1	9172	9268	96	93–104
2	9223	9403	180	155–201
3	9234	9261	27	15–40
4	9301	9316	15	1–25
5	9210	9216	6	−29–44
6	9196	9280	84	56–92
7	9157	9243	86	60–110

*Means for ten test specimens/group.
†Both initial and final weighings were made immediately after air-conditioning of the samples for 24 hours at 20 C and 65% relative humidity.

stant weight over phosphorus pentoxide, regains its initial weight when it is air-conditioned again, and, secondly, to define the way in which the release and absorption of water by a cardboard sample may be influenced by any HB 307. To this end, ten cardboard samples of the same origin were stored at 23 C and 50% relative humidity to constant weight. Half of the samples were dipped into HB 307, wiped and weighed immediately to determine the amount of test fat absorbed. Subsequently, all ten samples were stored for 30 days in a desiccator over phosphorus pentoxide, weighed and finally re-exposed to the storage conditions (23 /50%) until stabilized. Both types of sample released about the same amount of water (Table 8), but on subsequent air-conditioning neither regained the initial weight. In the case of the fat-free samples, the mean final weight was 10·7 mg (some 0·5%) below the weight after the initial storage, while for the fat-containing samples the mean decrease was 6·5 mg, or below 0·3%. Thus the test fat appears to have some effect on the reabsorption of water by the dried board.

If, in the investigation of a cardboard, the group 1 measurements were considered as a blank test, a blank value of 11 mg would have to be deducted from the total-migrate value found. However, in effect, due to the presence of the test fat, the blank value would amount to only 7 mg, and the total-migrate calculation would be wrong by 4 mg.

Since contamination of the cardboard by the test fat can hardly be avoided in the investigation of cardboard/plastics laminates, re-acclimatization of the stored sample to the original storage conditions with subsequent determination of the weight (Gn) cannot be recommended. A more promising approach is the determination of the dry weights of the sample before and after contact with the test fat. However, since the sample material will be changed during drying, it is not reasonable to determine directly the dry weight of the sample before its contact with the fat. The possibility of calculating the dry weights of starting samples with the aid of experimentally determined weight losses from corresponding blank samples was therefore investigated.

Initially, a fairly large batch of samples of a cardboard/HD-PE laminate was exposed at 23 C and 50% relative humidity to constant weight. After storage for 40 hours at 105 C and 12 mm Hg, the mean dry weight of a proportion of these samples (group 1) was determined and the mean percentage weight loss (water content) was calculated to amout to 3·10%. Using this percentage, we calculated the dry weights of three groups of samples from the mean weights of the samples immediately after storage at 23 /50% (groups 2–4; Table 9). The group-2 samples were dried for 40 hours at 105 C/12 mm Hg immediately after the determination of their initial post-storage weights just as with group 1, and the difference between the calculated and the actual dry weights was −1 mg for a total weight of 1595 mg. When the samples were stored for 10 days at 40 C/65% before the determination of the dry weight (group 3), the mean difference between the calculated and the actual weights amounted to −2 mg (total weight 1598 mg). With larger specimens pretreated in the same way and comminuted before drying (group 4) the mean difference was +2 mg (total weight 2622 mg).

Without identifying more closely this influence of the pretreatment of samples on the determination of their dry weights, it should be possible to eliminate the effect by the identical treatment of sample and blank, provided the test fat present under practical conditions does not exert a dominating influence on the determination of the dry weight. Therefore, in a further experiment carried out in accordance with those just described, we checked the influence of the test fat HB 307 on the determination of the dry weight of a cardboard/HD-PE laminate (Table 9).

The dry weight of the laminate was determined on

Table 8. *Influence of HB 307 on the amounts of water released and absorbed by cardboards*

Group no.	Mean weight (mg) of test specimens after		Water release during 4-wk storage‡ (mg)	Re-absorption of water during final storage* (mg)	Difference between initial and final weight (mg)
	Storage*	Application of HB 307†			
1	2312 ± 7·4	—	97·1 ± 1·8	86·4 ± 1·7	−10·7 ± 0·2
2	2308 ± 10·5	3531·0 ± 22·6	96·4 ± 0·4	89·9 ± 0·5	−6·5 ± 0·3

*At 23 C and 50% relative humidity, continued until two consecutive weighings carried out under identical atmospheric conditions and separated by a 24-hr interval differed by < 1 mg.
†At 23 /50%.
‡In a desiccator over phosphorus pentoxide (23 /0%).
Values are means ± standard deviations for groups of five samples.

Table 9. *Determinations of the absolute dry weight (TG) of a cardboard/HD-PE laminate and the influence of HB 307 on the dry-weight determinations*

Laminate determinations and treatment	Sample group no.	No. of samples	Mean weight (mg) of test samples after				Difference between TG calc. and found
			Storage*	Application of HB 307†	Drying‡		
					TG found	TG calculated§	
Water content of samples (3·10"„ w/w)	1	5	1641·0 ± 6·3		1590·0 ± 6·2		
Determination of TG							
Immediately after storage at 23 /50"„	2	5	1645·0 ± 6·3		1595·0 ± 6·7	1594·0 ± 6·1	− 1·0 ± 0·8
After storage to constant weight at 23 /50"„ and for 10 days at 40 :							
whole samples	3	5	1646·9 ± 6·6		1598·0 ± 6·4	1596·0 ± 6·0	− 2·0 ± 0·8
comminuted samples	4	3	2708·0 ± 8·2		2622·2 ± 7·9	2624·0 ± 7·9	1·8 ± 0·2
Water content of samples (3·13"„ w/w)	5	4	1662·0 ± 3·8		1610·0 ± 3·2		
Determination of TG							
Immediately after HB 307 application	6	5	1641·0 ± 5·3	1778·7 ± 10·2	1728·0 ± 10·4	1727·7 ± 10·1	− 0·3 ± 1·1
After application of HB 307 and 2-day storage at 23 /50"„	7	5	1637·5 ± 5·4	1782·9 ± 31·9	1732·0 ± 28·1	1731·0 ± 27·9	− 1·0 ± 0·6

HD-PE = High-density polyethylene

*At 23 C and 50"„ relative humidity, continued until two consecutive weighings carried out under identical atmospheric conditions and separated by a 24-hr interval differed by < 1 mg.

†As a thin film on the PE side of the laminate, applied in the liquid state at 23 /50"„.

‡At 105 C/12 mm Hg for 40 hr.

§From mean weight and water content of group-1 or group-5 samples stored at 23 /50"„.

Values are means ± SD for the stated numbers of samples.

a group of samples that received no treatment apart from the preliminary air-conditioned storage and therefore served as a blank (group 5). In two similar groups, a known amount of the test fat in the liquid state was applied as a thin film to the polyethylene side of each sample and the dry weights of the samples were calculated either immediately after application of the fat (group 6) or after further storage for 2 days at 23°/50% (group 7). In both of these groups the mean difference between the dry weight found and that calculated from the water content of the blank samples was low, indicating that the test fat had no significant effect on the dry-weight determinations.

Having checked in this way all the steps for the determination of the total migration of components from cardboard/plastics laminates into fat-releasing foodstuffs, it is worthwhile to examine the entire procedure, as follows:

(1) The weight of the sample before contact with the test fat is determined after constant storage at 23°C and 50% relative humidity (Gv expressed in mg).
(2) The weight of the sample after contact with the test fat is determined as the dry weight after storage for 40 hours at 105°C and 12 mm Hg (Gnt in mg).
(3) The water content a (weight loss) of the starting sample is determined on blanks stored together with the sample (aH_2O in Mg).

Thus the formula for the determination of total migrate from cardboard/plastics laminates reads:

$$GM = \frac{Gv - aH_2O - Gnt + Fp}{KF}$$

where KF is the contact area (dm^2) between test material and test fat and Fp is the amount of fat (mg) in the stored test material.

We have checked this procedure on samples of a cardboard/HD-PE laminate (Table 10). Determined with the test fat HB 307, the total migrate of the HD-PE film used for the laminate amounted to 1·3 mg/dm^2. In experiments 1, 2a, 2c and 2d the contact area KF between sample and test fat was 0·51 dm^2 and in experiments 2b, 2e and 2f it was only 0·264 dm^2. Moreover, as indicated in Table 10, the numbers of experimental samples and simultaneously stored blanks in each group varied.

Comparison of the total-migrate values obtained for the cardboard/HD-PE laminate under the same test conditions but with varying numbers and sizes of samples and blanks and their further comparison with the corresponding value for the HD-PE film cannot be considered encouraging at first sight, since whereas the total migrate for the HD-PE film amounted to 1·3 mg dm^2, the values for the cardboard HD-PE laminate lay between −1·1 and 17·2 mg dm^2. Nevertheless, experiments 2a and 2c show that with a contact area of 0·51 dm^2 and a sufficiently high number of separate determinations, the mean total-migrate value of the cardboard HD-PE laminate is in good agreement with that of the HD-PE film. However, since in other cases studied single values have been found to deviate within limits of the order of 10 mg/dm^2, it seems questionable whether, with cardboard/plastics laminates, so low a total-migrate level can be checked adequately using the method of differential weighing.

Investigations are continuing into this and other problems involved in determining the total migrate from cellulose-containing materials coated with plastics.

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Table 10. *Determinations of the total components migrating from a cardboard HD-PE laminate into foodstuffs using [^{14}C]HB 307 as the test fat*

Test material	Experiment no.	No. of test determinations	Contact area, KF (dm^2)	No. of blank samples stored*	Total migrate, GM (mg dm^2)†
HD-PE films‡	1	7	0·51	0	1·3 ± 0·36
Cardboard HD-PE laminate	2a	10	0·51	10	1·9 ± 1·00
	b	10	0·264	10	−1·1 ± 3·50
	c	15	0·51	16	0·5 ± 1·61
	d	6	0·51	6	5·0 ± 2·62
	e	16	0·264	16	4·1 ± 8·40
	f	6	0·264	6	17·2 ± 5·21

HD-PE = High-density polyethylene

*The mean water content of blanks (% H_2O) stored under the same test conditions was used to calculate the total migrate, GM.

†Means ± SD for the stated numbers of test determinations.

‡Approximately 200 μm thick.

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CONTRIBUTION A L'ETUDE DE LA METHODOLOGIE D'EVALUATION DE LA TOXICITE CUTANEE. I. TOXICITE PERCUTANEE—TESTS D'IRRITATION

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Résumé—Les auteurs passent en revue les différents aspects de la toxicité cutanée ainsi que les méthodes d'évaluation généralement employées. Ils décrivent les techniques qu'ils utilisent personnellement pour la détermination de la toxicité aiguë par voie percutanée et les différents tests d'irritation.

Summary—The authors review the different aspects of cutaneous toxicity as well as the methods of evaluation generally used. They describe the techniques they personally use for the determination of percutaneous acute toxicity and the different irritation tests.

Introduction

La notion de toxicité cutanée d'un produit, pour simple qu'elle paraisse, recouvre en réalité des aspects aussi divers que la toxicité aiguë par pénétration percutanée, l'irritation cutanée par application unique ou par applications itératives, les problèmes de sensibilisation et de photoréaction. Ce premier article traitera de la toxicité aigüe et de l'irritation. Les problèmes de sensibilisation et de photoréaction feront l'objet d'un deuxième mémoire.

Toxicité aiguë par pénétration percutanée

En ce qui concerne la toxicité aiguë, son expression la plus facilement interprétable est la dose létale 50, dont la détermination est cependant plus longue et aléatoire que pour les autres voies de pénétration des toxiques. En effet, pour cette voie, on ne peut procéder que par ajustements successifs, car le taux et la vitesse d'absorption dépendent, entre autres, de la structure des produits, de leur liposolubilité, du pourcentage de surface corporelle en contact avec le produit; la quantité absorbée ne dépend pas directement de la dose appliquée et ne peut donc être déterminée qu'à postériori.

La technique généralement appliquée pour déterminer la dose létale 50 par voie cutanée chez l'animal—le plus souvent le lapin—est celle proposée par Draize (Draize, 1944; Draize, Woodard et Calvery, 1944). Elle consiste à maintenir le produit à étudier en contact avec la peau de l'animal au moyen d'un manchon de caoutchouc aux extrémités renforcées, s'adaptant étroitement autour du tronc rasé de l'animal. On utilise, pour chaque dose, un nombre suffisant d'animaux des deux sexes, et, sur la moitié d'entre eux, on pratique des abrasions épidermiques longitudinales tous les 2 ou 3 cm sur la surface exposée; ces abrasions doivent être assez profondes pour pénétrer la couche cornée, mais ne doivent pas atteindre le derme (absence de saignement). Les extrémités du manchon sont plissées, permettant à la partie centrale de ballonner et de former ainsi un réservoir pour la dose appliquée. Le manchon est adapté sur l'animal qui est installé dans une position confortable mais immobile dans un casier pouvant contenir plusieurs animaux. S'il se produit une légère fuite du liquide hors du manchon, ce qui peut arriver durant les premières heures, on le recueille dans un récipient de verre et le réapplique. Les quantités de produit sont ajustées lors des expositions ultérieures pour permettre de calculer la dose provoquant la mort de 50% des animaux. Au bout de 24 heures, on retire le manchon et mesure le volume de substance non absorbée. On note immédiatement les réactions cutanées et les symptômes apparents d'intoxication. Les animaux sont ensuite gardés en observation pendant 2 semaines. C'est cette technique qui a été adoptée par la Food and Drug Administration des Etats-Unis (Regulations under the Federal Hazardous Substances Labeling Act, 1965; CFR Title 21, Chapter I, Part 191).

Nous trouvant de plus en plus souvent confrontés avec le problème de la toxicité cutanée des solvants industriels, manipulés parfois sans précautions par les ouvriers, nous nous sommes heurtés à certaines difficultés dans l'application de la technique de Draize,

en particulier la confection des manchons que l'on ne trouve pas en France dans le commerce. Il en est de même pour les casiers à animaux, qui présentent, en outre, l'inconvénient de maintenir l'animal immobile pendant 24 heures. Enfin, les fuites possibles, même si le produit ainsi écoulé est recueilli et réappliqué, entraînent des pertes qui ne sont pas toujours négligeables, surtout si les doses expérimentées sont faibles. Nous avons donc été amenés à modifier cette technique pour l'adapter aux besoins de nos expérimentations.

La méthode que nous avons proposée (Truhaut, Catella et Nguyen Phu-Lich, 1969) est basée sur le même principe que celle de Draize. Elle en diffère essentiellement par le fait qu'elle utilise un matériel facile à obtenir, élimine le risque de fuite, permet de calculer la quantité exacte de produit absorbée et laisse l'animal libre de ses mouvements dans sa cage.

Technique proposée

Dans un sac de polyéthylène, on introduit une bande de caoutchouc type 'feuille anglaise' d'environ 50 cm de long et 18 cm de large, un carré de feuille anglaise d'environ 17 cm de côté, un carré de coton cardé d'environ 15 cm de côté, une compresse de gaze de 10 cm de côté et une bande de gaze de 2,50 m de long et 7 à 10 cm de large. Le poids de l'ensemble est noté.

Les lapins, par moitié mâles et femelles, en nombre suffisant pour chaque dose, d'un poids voisin de 2,5 kg de préférence (sans excéder 3 kg), sont rasés sur le dos et les flancs sur une surface d'environ 250 cm^2, 24 heures avant l'expérience pour éviter l'interférence d'une irritation cutanée due à l'effet de la tondeuse. Sur la moitié des lapins, on pratique sur la surface destinée à être exposée des abrasions épidermiques longitudinales tous les 2 ou 3 cm. Les animaux sont laissés à jeun la veille de l'expérience.

On opère sur chaque animal de la façon suivante: sur la peau rasée on applique successivement la compresse de gaze, le carré de coton cardé et le carré de feuille anglaise, et on maintient le tout en place en faisant un bandage autour du tronc du lapin avec la bande de gaze. On introduit alors une quantité connue du solvant à étudier à l'aide d'une pipette dont on enfile l'extrémité sous la compresse de gaze, au contact de la peau. Pour assurer l'étanchéité, on entoure ensuite l'animal de la bande de feuille anglaise que l'on fixe au moyen d'agrafes, de telle façon qu'il soit confortable et libre de ses mouvements sans pouvoir se débarrasser du manchon ainsi formé. Le lapin est alors remis dans sa cage et garde ce dispositif pendant 24 heures. Au bout de cette période —ou sitôt la mort de l'animal si celui-ci a succombé avant—on le débarrasse de son manchon dont on remet tous les éléments dans le sac de plastique (ce qui évite toute évaporation du solvant) et on effectue une nouvelle pesée. Cette pesée étant effectuée immédiatement après la fin de l'essai, aucune perte par volatilisation due à un défaut d'étanchéité du sac en plastique ne peut se produire.

La différence de poids après et avant intoxication correspond à la quantité non absorbée par le lapin, cependant que la différence entre la quantité introduite et la quantité restante donne la dose réellement absorbée. Les réactions cutanées et les symptômes d'intoxication sont notés; les survivants sont gardés en observation pendant 2 semaines, puis sacrifiés et autopsiés. Les organes sont alors prélevés pour l'examen anatomo-pathologique.

On peut alors tracer la courbe du pourcentage de mortalité par la dose administrée selon une méthode classique, et déterminer ainsi la valeur de la DL_{50} cutanée du produit étudié.

Remarques

Quelques remarques nous paraissent nécessaires. Au coton hydrophile nous avons préféré le coton cardé qui ne retient que l'excès de liquide et le redistribue à mesure de l'absorption par la peau du lapin. La feuille anglaise a été choisie comme bande imperméable, en raison de sa légèreté et de son élasticité. Il est préférable de laisser l'animal à jeun la veille de l'expérience. En effet, l'élasticité de la feuille anglaise permet à celle-ci de se prêter à une dilatation stomacale lors de l'absorption de nourriture par le lapin; au contraire, si l'estomac était déjà dilaté avant l'expérience, la digestion entraînerait une diminution de volume et, le manchon gardant ses dimensions initiales, l'étanchéité ne serait alors plus assurée.

Discussion

Cette technique, facile à mettre en oeuvre et d'exécution rapide (avec un peu d'habitude, deux personnes, l'une maintenant le lapin, l'autre manipulant, pouvant traiter six lapins en une demi-heure environ), est plus particulièrement destinée à la détermination de la DL_{50} cutanée de solvants peu volatils. En effet, dans le cas de solvants très volatils, il pourrait y avoir une évaporation partielle et non mesurable aux extrémités du manchon. La technique de Draize ne paraît cependant pas meilleure à cet égard, car le liquide qui peut fuir durant les premières heures risque aussi de s'évaporer avant d'être recueilli et réappliqué.

Si, au lieu de laisser l'animal en contact avec la substance pendant 24 heures, on arrête l'intoxication au bout de 1, 2, 3 heures..., on peut déterminer la cinétique d'absorption cutanée du produit.

Appliquée dans les conditions indiquées, la méthode que nous proposons permet de déterminer, de façon satisfaisante, la vitesse de pénétration d'un liquide à travers la barrière cutanée et sa toxicité par cette voie de pénétration.

Si la toxicité percutanée d'un produit est le plus souvent appréciée en déterminant sa DL_{50} par cette voie chez le lapin, d'autres manifestations toxiques peuvent être prises en compte. Ainsi McCreesh (1965) détermine-t-il également une ED_{50} définie comme la dose la plus faible estimée produire une réaction chez la moitié des animaux testés, et une ET_{50} définie comme le temps estimé à partir duquel on constate une réponse toxique à une dose déterminée chez la moitié des animaux testés. Cet auteur n'indique cependant pas ce qu'il entend par "réaction" ou "réponse" toxique.

D'autre part, certains auteurs comme Noakes et Sanderson (1969) effectuent la détermination de la DL_{50} cutanée, non pas chez le lapin mais chez le rat.

Enfin, il est évident que l'absorption percutanée d'un produit et les différents facteurs qui la conditionnent ont une influence directe sur sa toxicité par cette

voie. Sans entrer dans le détail des nombreuses études consacrées à ce sujet nous citerons les revues de Wepierre (1969) et plus récemment de Idson (1975) faisant le point sur cette question.

Irritation cutanée par application unique (Irritation cutanée primaire)

En ce qui concerne l'appréciation du pouvoir irritant éventuel des produits sur la peau soit après une application unique, soit après des applications répétées jour après jour pendant une certain temps. la tâche du toxicologue est grandement facilitée depuis l'adoption par la législation française d'une technique officielle, dérivée de la technique de la FDA aux Etats-Unis (CFR Title 21, Chapter I, Part 191), qui avait été mise au point initialement par Draize (1944) et Draize *et al.* (1959) et faisant l'objet des arrêtés du 5 avril 1971 (*Journal officiel* du 21 avril 1971) et du 16 avril 1973 (*ibid* 5 juin 1973). Ces arrêtés ont été pris dans le cadre des méthodes officielles d'analyse pour les Cosmétiques et produits de Beauté (Répression des Fraudes), mais la technique est applicable à n'importe quel produit.

La technique officielle

L'essai est pratiqué sur six lapins albinos préalablement rasés sur le dos et les flancs et sur lesquels ont été pratiquées d'un côté de légères scarifications, l'autre flanc restant intact.

La technique consiste à déposer une quantité déterminée de la substance à tester sur deux carrés de gaze (0,5 g s'il s'agit d'un produit solide ou pâteux, 0,5 ml s'il s'agit d'un liquide), à appliquer ces carrés, l'un sur la peau scarifiée, l'autre sur la peau intacte des animaux et à les maintenir au contact de la peau pendant 24 heures à l'aide de fixations de sparadrap. Les carrés de gaze sont enlevés 24 heures après l'application.

Les réactions doivent être évaluées 30–90 minutes après l'enlèvement des carrés de gaze et de nouveau 48 heures plus tard (24 heures et 72 heures après l'application respectivement) sur la peau intacte et la peau scarifiée, selon les critères suivants:

Erythème et formation d'escarres:	
Pas d'érythème	0
Léger érythème (à peine visible)	1
Erythème bien visible	2
Erythème modéré à important	3
Erythème grave (rouge pourpre) avec formation de légères escarres (lésions profondes)	4
Formation d'oedème:	
Pas d'oedème	0
Très léger oedème (à peine visible)	1
Léger oedème (contours bien définis, gonflement apparent)	2
Oedème moyen (épaisseur environ 1 mm)	3
Oedème grave (épaisseur supérieure à 1 mm et surface supérieure à celle du carré)	4

La moyenne des notes ainsi obtenues est désignée comme étant '*l'indice d'irritation cutanée primaire*' (IIP) dont la valeur maximale est de 8.

D'après la législation française, la substance est considérée comme non irritante pour un indice nul, légèrement irritante pour un indice compris entre 0 et 2, moyennement irritante pour un indice compris entre 2 et 5 et sévèrement irritante pour un indice compris entre 5 et 8.

L'évaluation des érythèmes est facilitée en effectuant une injection intraveineuse, dans l'oreille du lapin, d'une solution à 1% de bleu trypan dans le sérum physiologique stérile, à raison de 1 ml de solution/kg de lapin. Les zones vascularisées se colorent en bleu et l'intensité de la coloration est proportionnelle à l'intensité de l'irritation.

Remarques

Cette technique peut, à notre avis, faire l'objet d'un certain nombre de critiques. Ainsi l'expérience nous a montré que l'emploi de sparadrap, même 'hypoallergique' entraîne toujours une irritation qui est gênante dans l'appréciation de la réaction cutanée due à l'application du produit. D'autre part, le coût de fabrication des harnais protecteurs de cuir souple destinés à empêcher les animaux de déplacer les carrés de gaze imprégnés est élevé pour un laboratoire effectuant simultanément plusieurs déterminations d'indice d'irritation cutanée primaire. Nous avons constaté qu'un rectangle de feuille anglaise placé à cheval sur le dos du lapin de manière à ce qu'il recouvre bien les deux carrés de gaze, bien fixé en faisant un bandage autour du tronc de l'animal à l'aide d'une bande de gaze est suffisant pour maintenir les carrés sans sparadrap, le harnais étant remplacé par un manchon de feuille anglaise dans les mêmes conditions que celles de la détermination de la DL_{50} cutanée (Truhaut, Dutertre-Catella et Nguyen Phu-Lich, 1972).

Il peut être intéressant de compléter l'examen macroscopique par un examen histologique des coupes de peau.

D'autres colorants que le bleu trypan peuvent être utilisés pour faciliter la lecture, ainsi Brown et Clarke (1965) ainsi que Wolven et Levenstein (1967) préconisent-ils l'emploi du bleu sulphan.

Discussion

Les deux principaux reproches que l'on puisse faire à cette méthode sont, d'une part, la subjectivité de l'observateur dans l'appréciation du degré d'irritation, d'autre part, les variations de sensibilité à un même produit de différentes espèces animales.

En ce qui concerne le premier reproche, Weil et Scala (1971) ont fait ressortir la divergence des résultats obtenus par 25 laboratoires différents qui avaient effectué des tests d'irritation cutanée et oculaire sur des produits provenant de lots identiques, et ont proposé d'instaurer des cours de recyclage pour réduire les différences d'évaluation individuelles. Notons à ce propos que ces auteurs, outre l'érythème et l'oedème, évaluent également la nécrose qui peut faire suite à l'application cutanée des produits testés.

Quant à l'espèce animale dont la réaction aux substances irritantes se rapproche le plus de celle de l'homme, de nombreux travaux ont été consacrés à ce sujet sans que les auteurs s'accordent toujours sur les conclusions. Si, comme Draize (1959), Levenstein (1964) préconise le lapin, Roudabush, Terhaar, Fassett et Dziuba (1965), dans une étude comparative de l'action irritante de différents produits sur la peau du lapin et celle du cobaye, concluent que ces deux espèces réagissent de façon pratiquement identique et qu'il n'y a pas de raison scientifique de préférer l'une

à l'autre. Cependant, Idson (1968) fait valoir que ces techniques sont plus valables pour des irritants sévères que pour des irritants moyens ou faibles, ce qui est confirmé par une étude comparative de la réponse de la peau de lapin et de la peau humaine à certains irritants effectuée par Phillips, Steinberg, Maibach et Akers (1971).

L'injection de colorants, comme nous l'avons vu plus haut, facilite l'appréciation dans le cas de produits moyennement ou faiblement irritants. Une étude très intéressante a été effectuée par Davies, Harper et Kynoch (1972), qui ont comparé le pouvoir irritant de sept produits différents sur la souris, le cobaye, le lapin, le porc nain, le porcelet, le chien, le babouin et l'homme. Ces auteurs montrent qu'il existe une variabilité considérable dans la réponse d'irritation des différentes espèces animales. Ils suggèrent de procéder aux évaluations en deux phases, la première utilisant le lapin qui s'est toujours révélé au moins aussi sensible que l'homme ou plus sensible que lui, ce qui permet d'éviter les fausses réponses négatives; dans la deuxième phase on pourrait utiliser une espèce ayant une réaction plus voisine de celle observée chez l'homme. Dans certains cas, l'une ou l'autre des deux phases, seule, pourrait s'avérer suffisante.

De toute façon, il est difficile, pour l'appréciation du pouvoir irritant d'un produit, de dissocier les résultats de l'application unique et ceux d'applications répétées que nous allons maintenant envisager.

Irritation cutanée par applications répétées

Une autre forme de toxicité cutanée peut être due au contact répété du produit avec la peau pendant une période plus ou moins longue. On évalue cette toxicité en déterminant 'l'indice d'agressivité par applications itératives' (IAAI). La technique de détermination de cet indice a été codifiée dans l'arrêté du 5 avril 1971 (*Journal officiel* du 21 avril 1971) et dérive de la technique initialement proposée par Draize (Draise 1944; Draize *et al.* 1959).

Technique officielle

L'essai est pratiqué sur trois lapins albinos préalablement rasés sur le dos et les flancs sur une surface d'environ 80 cm^2/kg et sur une petite surface de 5 cm^2 sur l'arrière train, servant de témoin.

La substance à tester est appliquée chaque jour, à la dose de 2 g de produit/kg de poids vif, sur toute la surface tondue, à l'exception de la zone témoin, de manière bien uniforme et en faisant bien pénétrer le produit à l'aide d'une spatule en plastique.

La durée de l'essai est de 90 jours. Les lapins sont tondus sur la surface d'application et la zone témoin une fois par semaine, ou plus si nécessaire. On note chaque semaine le poids des animaux, on observe l'aspect et la repousse des poids, on mesure l'épaisseur de la peau et l'hyperacanthose éventuelle et on recherche et note la formation d'érythème et d'oedème comme pour une mesure d'irritation primaire.

Il est souhaitable de procéder à un examen histologique de la peau et à une analyse de sang et d'urine.

Remarques

Une erreur évidente s'était glissée dans la technique décrite au *Journal officiel*. Celui-ci reproduisait en effet la notation utilisée pour déterminer l'indice d'irritation primaire, c'est-à-dire en additionnant les notes obtenues pour l'érythème et pour l'oedème après 24 et 72 heures, sur la peau intacte et sur la peau scarifiée, alors que dans les cas de l'appréciation de l'agressivité superficielle par applications itératives on ne pratique pas de scarifications et que la lecture se fait chaque semaine. Elle a été rectifiée par l'Arrêté du 17 janvier 1973 (*ibid* 9 février 1973), qui abroge ce paragraphe 'Notation'.

Il arrive parfois que l'on n'observe aucune irritation cutanée par applications itératives d'un produit qui avait entraîné une certaine irritation primaire. Mais ceci n'est paradoxal qu'en apparence car l'IIP est un test 'occlusif' où le produit reste enfermé au contact de la peau, c'est-à-dire isolé de l'air par un pansement imperméable, tandis que l'IAAI est un test 'ouvert', où le produit est mis au contact de la peau sans pansement isolant.

Discussion

Comme nous l'avons déjà souligné, il est difficile de dissocier les résultats des différents tests d'irritation par application unique et par applications répétées, qui sont complémentaires et doivent être associés pour l'interprétation. En effet, comme le fait remarquer Idson (1969), il est parfois malaisé, après une application unique, de distinguer une irritation primaire d'une sensibilisation. D'autre part, certains auteurs décrivent des tests de détermination de l'irritation cutanée 'primaire' où ils pratiquent des applications répétées plusieurs jours de suite, comme Uttley et Van Abbé (1973), qui appliquent la substance à étudier pendant 4 jours successifs sur l'oreille de la souris.

De nombreuses méthodes ont été proposées pour évaluer le pouvoir irritant de produits se situant en particulier dans la zone inférieure de l'indice de Draize (1959). Ainsi Finkelstein, Laden et Miechowski (1963 et 1965) combinent la technique d'injection de bleu trypan avec une présensibilisation au formol et des tests occlusifs sur différentes espèces animales. Opdyke et Burnett (1965) immergent des cobayes jusqu'aux aisselles dans les solutions à tester pendant 4 heures, 3 jours de suite! Ils laissent ensuite les animaux au repos pendant 2 jours puis notent l'irritation d'après l'aspect et la palpation. Justice, Travers et Vinson (1961) utilisent la combinaison de deux tests d'applications répétés: d'une part, sur l'oreille de souris où le nombre d'expositions est fixe et l'effet mesuré par examen histologique de la peau, d'autre part, immersions du bras humain et mesure du nombre d'expositions nécessaires pour obtenir une irritation donnée. Ce second test n'est mis en oeuvre que si le produit n'a pas provoqué d'irritation significative de l'oreille de souris.

Il est bien évident comme le soulignent, entre autres, Rieger et Battista (1963) ou Skog (1963), qu'en dernier ressort, les tests sur des volontaires humains sont naturellement les plus valables, à condition d'être pratiqués sur un assez grand nombre de sujets. Ceci

implique, bien sûr, d'avoir éliminé par l'expérimentation animale les produits les plus irritants.

C'est également sur des sujets humains qu'opère Kligman (1967), qui, pour éliminer les erreurs dues à des différences d'interprétation dans le degré d'irritation, propose une technique où la réaction se mesure par une réponse de 'tout-ou-rien', l'observateur n'ayant qu'à décider s'il existe ou non une réaction, sans mesurer le degré de celle-ci. Cet auteur, qui se refuse à faire une distinction entre une irritation 'primaire', produite par une seule application, et 'secondaire' produite par des applications répétées, détermine simplement si un irritant est 'fort' ou 'faible' en calculant deux valeurs caractéristiques: l'ID_{50} et IT_{50}, respectivement. Pour un irritant fort, l'ID_{50} est la concentration, statistiquement calculée, nécessaire pour produire une irritation décelable chez 50% de la population. Pour un irritant faible l'IT_{50} est le nombre de jours, statistiquement calculé, d'exposition continue qui produit une réaction seuil chez 50% de la population. Kligman (1967) souligne bien que ces valeurs peuvent varier selon les sujets testés (âge, sexe, race) et d'autres facteurs, comme la saison, par exemple, et ne prennent toute leur signification que par comparaison à un étalon de référence. Ces déterminations sont relatives et non absolues, l'utilité du test dépendant du choix d'un produit de référence approprié pour la comparaison.

Enfin, nous ne quitterons pas le domaine des applications cutanées répétées sans rappeler que le badigeonnage quotidien de l'oreille de souris pendant une période suffisamment longue est la méthode de choix pour révéler les potentialités cancérogènes cutanées d'un produit.

Conclusion

Nous voyons que l'étude de la toxicité aiguë par voie percutanée, destinée surtout à révéler les effets systémiques des produits pénétrant par cette voie et l'étude des phénomènes d'irritation cutanée destinée à révéler des effets locaux, sont en réalité indissociables. En effet, une dose massive peut également engendrer des effets d'irritation locaux, alors que des applications répétées, en altérant la barrière cutanée peuvent aboutir à des altérations graves de certains organes et même parfois à l'effet léthal. Il est donc important de soumettre les animaux parallèlement à ces deux types de tests, en utilisant des méthodes codifiées.

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BOOK REVIEWS

The Genetics and Biology of Drosophila. Vol. 1b. Edited by M. Ashburner and E. Novitski. Academic Press Inc., (London) Ltd., London. 1976. pp. li +468. £16.80.

The development of the science of genetics owes much to Drosophila. Not only was this insect the first organism from which mutants were isolated (by H. J. Muller in 1928), but it was also used to demonstrate, for the first time, that mutation can be induced by certain chemicals.

Drosophila has numerous advantages over other animals for the study of genetics. Most notable among these is its possession of giant, salivary gland chromosomes, detailed maps of which are available and which permit the detection of minute chromosomal rearrangements relatively easily. In fact, we know far more about the genetics of Drosophila than of any other 'higher' organism, which is one of the reasons why it provides a very useful method for the detection of environmental mutagens, one of the very few mutagenicity tests involving a multicellular organism that can be performed relatively quickly and cheaply.

In view of the indispensibility of this organism as a tool in genetics research it is rather surprising that the present volumes are the first attempt in some 50 years to present a comprehensive coverage of the subject. This book is only the second in a series of five, the first three of which (Vols 1a, b, c) are devoted to the genetics of Drosophila, while the fourth (Vol. 2) deals with its biology and development and the fifth (Vol. 3) with evolution and ecology, so the subject is being covered not only comprehensively but also in considerable depth. The chapters in this volume review various specialized aspects of Drosophila genetics and hence will probably appeal more to the researcher in Drosophila than to the general biologist. The book is impressively indexed with no less than four compilations including, in addition to those for authors, subjects and species, an invaluable index of genetic variations which lists all the Drosophila mutants and chromosome aberrations.

Immunology of the Gut. CIBA Foundation Symposium 46. Edited by R. Porter and J. Knight. Excerpta Medica, Amsterdam. 1977. pp. viii +376. $28.00.

This book consists of a collection of invited papers given at a CIBA Foundation Meeting in April 1976. The meeting was held in memory of the late Joseph Heremans, a scientist who did much to establish the structure and function of the IgA class of antibody.

Each contribution has been carefully selected so that the book presents a complete review of the current state of gut immunology. The papers in the first section deal mainly with the protective role of the gut-associated immune system while those in the second section are devoted to an appraisal of gastrointestinal diseases that are considered to have an immunological aetiology.

The role of IgA, naturally enough, receives full coverage, with contributions on the natural history of IgA-secreting cells and the role of Peyer's patches. A paper by P. Brandtzaeg and K. Baklien is particularly interesting in this respect, presenting a hypothetical model for the secretion of IgA and IgM and considering their protective role against bacterial colonization and food proteins. There is also some discussion as to whether IgE can be thought of as a secretory immunoglobulin with a physiological function analogous to that of IgA. T. Lehner provides an adequate discussion of the immunological responses to oral plaques and presents the results of his studies on cell-mediated immune responses to oral bacteria and their products.

Two papers consider the immunological consequences of nematode infection. In the second of these, B. M. Ogilvie and D. M. V. Parrott present evidence for the participation of both circulating antibodies and sensitized lymphocytes in the allergic response against such infections; they also claim, rather surprisingly, that IgE antibodies play no part in parasite rejection.

Papers relating more specifically to the immunopathology of inflammatory bowel disease are concerned with model systems designed to assess the local effects of delayed hypersensitivity on the structure and function of the small intestine. The pathological sequelae of immunodeficiency are well reviewed by A. J. Katz and F. S. Rosen and alpha chain disease is singled out for special attention by M. Seligmann.

This book can be highly recommended. The discussion sections at the end of each paper are worth reading in addition to the general discussion at the end. The quality of production is good and the presentation is certainly suitable for the non-specialist.

An Introduction to General Pathology. By W. G. Spector. Churchill Livingstone. Edinburgh. 1977. pp. viii +316. £3.25.

This book is a refreshing departure from the 'dry-as-dust' style of most pathology text-books. It deals with basic concepts of the origin and causes of disease rather than with a collection of descriptions of morphological changes strung untidily together.

The opening chapter, a thoughtful essay on the meaning of pathology, stresses the fact that to understand pathology one must understand certain recurrent themes that underlie disease processes. The four major themes identified by the author deserve mentioning not only because of their intrinsic interest but also because they are an example of the philosophy

that pervades this book. The first is that disease often originates from a perversion of a survival mechanism; antibodies, for example, combat pathogenic organisms but may also cause autoimmune disease. Secondly, failures of adaptation tend to be self-reinforcing and progressive, a point reflected in the failure of organ function as a result of chronic progressive disease. The third theme is that quick responses to unfavourable environmental events are often overdone, as in the development of dehydration and the loss of sodium from excessive sweating, and finally it is clear that pathological duels, such as those between bacteria and man, tend to be fought to a draw rather than to outright victory. In connexion with the latter point the author maintains that "the reason for this paradox is that natural selection favours such a conclusion for both parasite and host".

However, the book as a whole is far from a philosophical treatise on pathology. It is written in simple but sound English, every technical term is clearly defined and the style holds the attention and interest of the reader. The wealth of pictorial diagrams adds considerably to the clarity of the text, making the book a valuable introduction for those coming into contact with pathology for the first time. It should also prove of interest to the not-so-young pathologist who feels the need to keep in touch with the changing scope of his subject. The chapters dealing with the immune system in disease and with the systemic response to injury are good examples of this aspect.

This book may be thoroughly recommended also to all those interested in toxicology. Much of the author's thinking about pathological processes and the explanations put forward as to their causes may throw light on the many puzzling phenomena encountered in animal tests. Moreover, anyone with a basic knowledge of biology should be able to follow and understand the ideas that the author is trying to impart.

Chalones. Edited by J. C. Houck. North-Holland Publishing Company, Amsterdam, 1976. pp. xiii +510. Dfl. 165.00.

Few concepts in the biological field have raised as much controversy as the chalone hypothesis put forward by W. S. Bullough in 1962. The resulting self-consciousness among some chalone workers is reflected in the cautionary tone of this book's introduction and the inclusion of a chapter headed "Critique" at the end.

The Bullough hypothesis was that the numbers of cells in a tissue are maintained in equilibrium by a negative feedback control mechanism mediated by tissue-specific mitotic inhibitors (chalones) produced by cells of the same tissue. The criticisms of this hypothesis have stemmed mainly from the simple chalone extraction procedures used by many workers and from the inadequacy of some of the assay systems used to demonstrate their activity. Furthermore, the finding of bacterial spores in a 'chalone' extract from a melanoma, reported by U. Mohr *et al.* in 1972, prompted the suggestion that the presence of bacteria or their toxins in tissue extracts might be responsible for many of the cases of mitotic inhibition attributed to chalone activity. In spite of these doubts, this book shows that the circumstantial evidence for the existence of chalones is strong, and it seems unlikely that bacterial activity or other toxic effects could provide an adequate explanation for the results obtained. The requirement for adrenalin by epidermal G2 chalone is an example of one instance where a toxic effect could not be presented as an alternative explanation.

Included in this book are three chapters dealing with epidermal chalone systems in relation to normal growth control and their role in neoplasia. The first of these chapters, by W. S. Bullough and E. Mitrani, is separated from the other two, its position at the beginning of the book probably reflecting the roles of both the epidermis and the first author in the development of the chalone concept. Between this and the other chapters on epidermal chalones are contributions on the history of chalones, the cell cycle, assay methods and the regulation of cell growth. These chapters give an excellent insight into the background of the methods used in chalone research and will be invaluable for those commencing work in this field. The rest of the book describes progress made in the demonstration of chalones specific to various other tissues or types of cell, including those associated with fibroblasts, melanocytes, granulocytes, lymphocytes, bronchial tissue, the liver and ascites tumours, and considers chalone involvement in colon carcinoma and the tissue specificity of chalones in relation to the embryonic derivation of tissues.

Clearly the development of chalone research has reached a stage where a breakthrough is required. The Editor of this volume leaves us in no doubt that he considers the purification and characterization of a chalone to be the breakthrough required to confound the sceptics and open up new avenues for research.

BOOKS RECEIVED FOR REVIEW

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Miscellaneous Pharmaceutical Substances. Vol. 13. International Agency for Research on Cancer, Lyon, 1977. pp. 255. Sw.fr. 30.00 (available in UK from HMSO).

Clinical Chemistry and Chemical Toxicology of Metals. Proceedings of the First International Symposium organized by the Commission on Toxicology. IUPAC Section on Clinical Chemistry, held at Monte Carlo, 2–5 March, 1977. Edited by S. S. Brown. Elsevier/North Holland Biomedical Press, Amsterdam, 1977. pp. xi +398. Dfl. 96.00.

Second Meeting on Mycotoxins in Animal Disease, Aberdeen 1976. Edited by D. S. P. Patterson, G. A. Pepin and B. J. Shreeve. Agricultural Development and Advisory Service. Ministry of Agriculture, Fisheries and Food, Pinner, 1977. £2.00.

Toxicology Annual, Volume 2. Edited by C. L. Winek and S. P. Shanor. Marcel Dekker, Inc., New York, 1977. pp. xii +272. Sw.fr. 98.00.

The Macrophage in Neoplasia. Edited by M. A. Fink. Academic Press Inc. (London) Ltd., 1976. pp. ix +265. £8.85.

Experimentation in Biology. An Introduction to Design and Analysis. By W. J. Ridgman. Blackie & Son Ltd., Glasgow, 1975. pp. ix +233. £4.20.

PCB Poisoning and Pollution. Edited by K. Higuchi. Academic Press Inc. (London) Ltd., 1976. pp. vii + 184. £12.40.

International Agency for Research on Cancer. Annual Report 1976. IARC, Lyon, 1976. pp. 159. Sw.fr. 12.00 (available in UK from HMSO).

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Asbestos. Vol. 14. International Agency for Research on Cancer, Lyon, 1977. pp. 106. Sw.fr. 14.00 (available in UK from HMSO).

Writing a Scientific Paper. 4th Ed. by V. Booth. The Biochemical Society, London, 1977. pp. 32. £1.00.

Fd Cosmet. Toxicol. Vol. 16, pp. 187–190 Pergamon Press Ltd 1978. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

THE SMOKING MOTHER REVISITED

In our last review of the effects of smoking on mother and foetus (Cooper, *Fd Cosmet. Toxicol.* 1975, **13,** 387) we concluded that, on the evidence then available, several different factors in the mother's environment may impinge on the perinatal stage of reproduction and have a harmful influence on the infant and growing child. This conclusion is reinforced by evidence that has since appeared.

General aspects

In a leading article, the *British Medical Journal* (1976, **2,** 492) has stated that carbon monoxide (CO), nicotine and cyanide (CN) are the factors in tobacco smoke that are most likely to have an adverse effect upon a foetus. The foetal hypoxia caused by CO may be aggravated by the ability of nicotine to reduce the breathing movements of the foetus, while the raised thiocyanate (SCN) levels reported in the cord blood of smoking mothers possibly represent a defect in CN detoxication associated with the lower vitamin B_{12} (cyanocobalamin) levels also found in smokers. Moreover, a depressed appetite, which is commonly a result of smoking, may impair foetal weight by reducing the mother's calorie intake.

To neglect the influence of the smoking father could be to ignore another possibly significant factor, in view of a report by Mau & Netter (*Dt. med. Wschr.* 1974, **99,** 1113). In a study of the smoking habits of fathers of 5200 infants, an increase in perinatal mortality was demonstrated when the father smoked more than ten cigarettes daily, even when the mother was a non-smoker. This effect was independent of the age of the mother or father, the socio-economic status of the family, and the numbers of live births, previous miscarriages or abortions and previous stillbirths. When the father was a heavy smoker (smoking more than ten cigarettes daily) the incidence of infants with severe malformations was roughly double that in the group with non-smoking fathers. Neural-tube defects, gross facial lesions, genito-urinary malformations and heart defects were among the aberrations recorded.

An analysis of 1159 mother-infant pairs by Davies *et al.* (*Lancet* 1976, **I,** 385) indicated that non-smoking mothers gained significantly more weight than those designated heavy smokers (more than 15 cigarettes daily) or light-to-moderate smokers (1–14 daily). Birth weight, body length and head circumference in infants all followed a similar gradient in relation to the degree of smoking. These authors suggested that the use of suitable dietary supplementation to increase weight gain to a rate similar to that found in non-smokers might offset the harmful effects of maternal smoking on foetal growth and development. Mau (*ibid* 1976, **I,** 972) disagreed, however, maintaining that a German prospective study on pregnant women who were non-smokers or light, intermediate or heavy (more than 10 cigarettes daily) smokers had shown no significant association between smoking and weight gain during pregnancy. The number of babies calculated as being too light in relation to duration of pregnancy, body length and head circumference was not increased when the mothers smoked. However, a close correlation appeared between the number of small-for-dates babies and smoking habits in a group of women who gained weight normally during pregnancy. These German findings, suggesting that the birth of small babies to smoking mothers is not a simple question of malnourishment, were confirmed by a French study reported by Spira & Servent (*ibid* 1976, **I,** 1416), in which no relation could be established between maternal weight gain and smoking habits during pregnancy, although smoking reduced infant birth weights.

Attempts to explain the sudden infant death syndrome (SIDS) in terms of the smoking habits of mothers have been complicated by other factors. Bergman & Wiesner (*Pediatrics, Springfield* 1976, **58,** 665) compared 56 Seattle families who had lost infants by SIDS with 86 control families. They found that a higher proportion of SIDS mothers than of the control group smoked during pregnancy (61 against 42%) and after the birth of their infants (59 against 37%) and among smokers in both groups there was a higher consumption of cigarettes among the SIDS mothers. In Ontario during 1960–61 perinatal mortality was found to be increased significantly among the infants of smoking women, but not in a simple linear fashion (Meyer *et al. Am. J. Epidemiol.* 1974, **100,** 443), being significantly affected by a variety of other risk factors. The increase in risk of perinatal mortality in the families of smokers compared with non-smokers was calculated as ranging from less than 10% among young, low-priority, non-anaemic light smokers to 70–100% among heavily smoking mothers of high parity and relatively low socio-economic status, with a history of previous low birthweights or a low haemoglobin level (below 11 g/100 ml).

Role of smoke constituents

Manning *et al.* (*Br. med. J.* 1975, **1,** 552) demonstrated that in 18 habitual smokers at weeks 32–38 of uncomplicated pregnancies, the smoking of two cigarettes in succession reduced the period during which foetal chest movements could be detected between periods of apnoea. The maximum effect

occurred about 35 minutes after the start of the smoking, when the movements occurred for only 45% of the time compared with 65% in the pre-smoking (control) period. This effect was attributed, tentatively, to the pharmacological action of nicotine. Asmussen & Kjeldsen (*Circulation Res.* 1975, **36,** 579) studied umbilical cords from neonates from 15 non-smoking and 13 smoking mothers. Electron microscopy demonstrated pronounced changes in the arterial intima in the cords from smokers. The main degenerative changes observed were swelling, blebbing, contraction and subsequent opening of the endothelial junctions and formation of sub-endothelial oedema, together with dilatation of the endoplasmic reticulum of the endothelium and widening of the basement membrane. Similar degenerative changes have been seen in the arteries of animals exposed to CO or perfused with nicotine.

A study of Cardiff mothers who smoked showed that the number of cigarettes smoked daily and the weight of tobacco consumed increased during the period 1965–74 but the tar and nicotine intake fell (Newcombe. *Br. med. J.* 1976, **2,** 755). During the same period the smoker's excess risk of perinatal loss increased and the birth-weight deficit fell. The figures suggest that the outcome of smoking during pregnancy may be more closely related to CO or CN intake than to tar or nicotine. Dow *et al.* (*ibid* 1975, **4,** 253) reported that the increase in carboxyhaemoglobin (COHb) concentration in the blood after the smoking of a single cigarette was 3·9% in pregnant women compared with 2·1% in non-pregnant ones, and was especially pronounced (5%) in the presence of anaemia. Since COHb concentration was inversely related to Hb concentration, it was argued that the foetus of an anaemic smoker might be at special risk from CO intoxication. In criticism, Ashton (*ibid* 1976, **1,** 42) pointed out that this study was confined to women in the second trimester of pregnancy, and to subjects who were instructed to puff every 40 seconds, with deep inhalation, to a total of ten puffs per cigarette smoked, whereas in real life normal smoking follows a different pattern. When ten pregnant women smoked one standard tipped cigarette in their normal fashion, the number of puffs per cigarette fell from 14·66 at 12–16 weeks to 11·4 at 33–40 weeks, rising again postnatally to 12·9. The same subjects showed no significant change in the dose of nicotine they derived from their cigarettes during different stages of pregnancy. A few determinations of blood COHb showed that the rise in COHb induced by a cigarette became less as the pregnancy progressed, from 3·17% at 12–16 weeks to 1·09% at 33–40 weeks, with a postnatal rise to 1·80%. Thus, asserted Ashton (*loc. cit.*), women alter their smoking behaviour during pregnancy and it is unsound to extrapolate data derived from a structured smoking regimen.

Pettigrew *et al.* (*Br. J. Obstet. Gynaec.* 1977, **84,** 31) have reported that in a group of 45 mothers the mean birth weight of babies born to women smoking more than 15 cigarettes daily was significantly lower than that of the babies of non-smokers (3·33 kg compared with 3·61 kg). These changes correlated with a higher whole-blood concentration of CN in smokers, and with a raised concentration of SCN in the plasma and urine of smoking mothers at 28, 32 and 36 weeks. The plasma-SCN concentrations in these smokers remained significantly higher at the time of delivery.

While it must be recognized that studies in this field suffer from the deficiencies common to most epidemiological work, there is no cause for complacency in any of these new findings, however complex the interactions of environmental factors connected with tobacco smoking may appear and however contradictory the reports may sometimes seem.

[P. Cooper—BIBRA]

MORE ABOUT ACRYLAMIDE

Reports of acrylamide poisoning resulting in man from occupational or other exposure have been backed by a series of studies in experimental animals within the last decade. These have demonstrated the neurotoxicity of acrylamide in rats and several other species and described the conditions under which the peripheral neuropathy characteristic of acrylamide intoxication develops and persists (*Cited in F.C.T.* 1977, **15,** 154). Some additional reports of industrial exposure and of further studies in rats have been published recently.

A case of acrylamide poisoning following industrial exposure is described by Davenport *et al.* (*Neurology, Minneap.* 1976, **26,** 919). The man was admitted to hospital with loss of sensation and unsteady gait after working with acrylamide for 6 months. Although he had used protective clothing he suffered from intense irritation and ulceration of the palms of his hands and soles of his feet. Other effects included anorexia, ataxia, clumsiness, slurred speech, trouble with swallowing, a decrease in muscle tone, impaired control of voluntary movements, tremor of the upper extremities and other sensory impairments. His haematology, and blood and urine chemistries were normal. His condition remained virtually stable during a 2-month period, but during the course of a year or so he recovered almost completely, retaining only mild neuromuscular impairment in the ankles. The occurrence of articulation problems and disproportionate ataxia of stance and gait suggested the possibility of central (brain stem or cerebellar) lesions in addition to the peripheral neuropathy. An earlier report (*Cited in F.C.T.* 1977, **15,** 154) demonstrated the possibility of mental disturbance as well as peripheral neuropathy following ingestion of high doses of acrylamide in domestic drinking-water.

In the industrially exposed patient, the main histological findings in a sural nerve biopsy were diffuse fibrosis and loss of nerve fibres, and enlarged axons both with and without myelin sheaths. Numerous

axons packed with bundles of fine filaments were apparent under the electron microscope. Some of these bundles had lost their longitudinal orientation and there were few distinguishable neurotubules in these regions. There was an increase in the number of small mitochondria and dense bodies in the affected tissue.

Further case histories of acrylamide poisoning, this time among a group of six workers in the construction industry (Kesson *et al. Post-grad. med. J.* 1977, **53,** 16), report similar sensory and motor effects to varying degrees, and add that in two of the patients, exposed to acrylamide for 29 and 22 weeks, there was little sign of recovery some 15 months after termination of exposure. Apparently no nerve biopsies were carried out on these patients.

An experimental study of acrylamide poisoning in rats was made by Gipon *et al.* (*Neuropathol. appl. Neurobiol.* 1977, **3,** 115). Rats given 50 mg acrylamide/kg/day ip showed a lower rearing frequency after only one dose; two doses also reduced walking activity and this stayed low for the 9 days of the test. Steady decrease in rearing activity preceded ataxia of the hind paws and splaying of the hind limbs preceded signs of muscle weakness. Administration of 20 mg acrylamide/kg/day ip for 9 days had little effect on activity, although on the final day there was significantly less rearing activity among this group than among the controls. The ability of the rats to balance on a rotating rod was changed significantly by a cumulative dose of 300 mg/kg acrylamide given in daily doses of 50 mg/kg and by a cumulative dose of 380 mg/kg given in daily doses of 20 mg/kg. Performance on the rotarod was normal after 10–12 days recovery, showing the reversibility of acrylamide poisoning.

Edwards & Parker (*Toxic. appl. Pharmac.* 1977, **40,** 589) criticized the rotarod method of assessing the disability resulting from a neuropathy in that it involves both memory and shock avoidance (since rats are discouraged from making contact with the floor below the rod by an electric shock). It is also time-consuming and requires complex apparatus. They (Edwards & Parker, *loc. cit.*) therefore developed an alternative quantitative method for assessing the degree of disability associated with acrylamide-induced peripheral neuropathy in rats. The method, based on the measurement of the hindfoot spread on landing in rats dropped onto a bench in a standard procedure, demonstrated a significant increase in limb spread after only three doses of 50 mg acrylamide/kg given ip 3 times/week or after 14 days on a diet containing 200 ppm or 7 days on a 400-ppm diet. The spread continued to increase for 7 days after the last ip dose of acrylamide was given and then decreased, being essentially normal by day 16 of the withdrawal period. A similar pattern followed oral dosing and withdrawal. Perhaps the main advantage of this method, which is not suitable in cases of very severe disability, is its simplicity, since the rotarod does apparently give an adequate indication of motor performance. Further work on the landing foot-spread technique is required to establish its general applicability to other neurotoxic compounds.

Slight reductions in food intake, body growth and faeces production were found in rats given 20 mg acrylamide/kg/day for 22 days (Gipon *et al. loc. cit.*). Urine production increased in these rats, probably because of a relatively high water intake. The 50 mg/kg/day dose rate led to a marked reduction in growth in parallel with a lower food intake and faecal output but had no effect on water intake.

The same study entailed histological and histochemical examination of 14 rats given 50 mg acrylamide/kg every other day and six controls. The test animals were killed after cumulative doses of 200, 400 or 550 mg/kg. Intramuscular motor nerve fibres were unaffected by 200 mg/kg, but at 400 mg/kg occasional motor nerve terminals seemed blunt and slightly swollen and after 550 mg/kg there were many motor nerve fibres and terminals with irregular swellings, although others seemed normal. The first signs of degeneration of peripheral nerves were seen at 400 mg/kg, occasional large nerve fibres being affected. After 550 mg/kg some 50–70% of the large nerve fibres and a few small fibres were involved. There were no histological findings in the spinal cord, and enzyme histochemistry was normal.

The authors argue that behaviour such as walking activity and rearing are not dependent only on peripheral nervous function, but reflect the animal's general well-being. At the high (50 mg/kg) daily dose level, reduction in activity occurred from the second injection, but rotarod performance was not impaired until a cumulative dose of 300 mg/kg had been reached. Thus the early decrease in locomotor activity is probably not a result of peripheral nerve changes. The changes in food and water consumption and the effects on excretory products indicate that acrylamide acts outside the sphere of peripheral nerves. The changes in urine output suggest a disturbance in water balance. Previous reports of bladder distension accompanying acrylamide poisoning (Fullerton & Barnes, *Br. J. ind. Med.* 1966, **23,** 210) tie-up with this, and are consistent with an autonomic neuropathy (McCollister *et al. Toxic. appl. Pharmac.* 1964, **6,** 172). The retardation of growth could only partly be explained by the lower food consumption, but the other mechanism involved has so far remained unidentified. Relevant in this context is the work of Edwards (*Br. J. ind Med.* 1975, **32,** 31), showing that an analogue of acrylamide, methylene-bisacrylamide impaired body growth although it did not cause neuropathy. These authors concluded that acrylamide did not act exclusively on the nervous system.

Continuing the work reported by Gipon *et al.* (*loc. cit.*) the same group (Schotman *et al. Neuropathol. appl. Neurobiol.* 1977, **3,** 125) attempted to unravel the pathological mechanisms responsible for the distal axonal degeneration observed in polyneuropathies by studying changes in the rate of incorporation of leucine into proteins of the spinal cord and brain stem during acrylamide intoxication. Distal axonal degeneration of peripheral nerves was induced in rats by a series of ip acrylamide injections and biochemical investigations were carried out during three phases of the disease: the latent phase before signs of sensory or motor-neuron deficits or abnormal histological findings were detectable, the stage when neuropathy was established, being marked by ataxia and minor changes in the terminal motor nerves, and the recovery period after ataxia had disappeared.

During the first two phases there was a marked depression in the incorporation of the radioactive label of the tritiated leucine into spinal cord and brain stem proteins. The decrease in incorporation was about 12% in the latent phase and reached about 20% when neuropathy was established. However, in the recovery period the incorporation rate was some 11% higher than that in the controls. Heart muscle was affected by a similar depression of incorporation during the middle phase of established neuropathy, but was normal during the recovery period, suggesting that the increase in incorporation into nervous tissue at this stage is more specifically concerned with the processes of repair, although it is possible that regeneration in the heart follows a different time course. Schotman *et al.* (*loc. cit.*) were also able to show that the reduction in incorporation was not the result of the lower food intake or body weight.

It was demonstrated by these workers *in vitro* that direct addition of acrylamide to tissue slices did not produce any significant change in the incorporation rate of leucine into proteins. This finding was to be expected if acrylamide toxicity *in vivo* developed slowly or via a metabolite. However, sections of spinal cord from rats treated with acrylamide *in vivo* did not show any changes in *in vitro* leucine incorporation. *In vitro* systems therefore gave no indication of whether the action of acrylamide is direct or indirect: this may result from inadequate functioning of the *in vitro* system.

Less labelled proteins were found in the anterior horn cells of treated animals before axonal degeneration than in those of controls. This difference could be explained by changes in the compartmentation of amino acids as a result of acrylamide intoxication as well as by changes in protein synthesis. However, these results, like those of earlier studies (*Cited in F.C.T.* 1974, **12**, 579), suggest a relationship between a disturbance in protein metabolism and acrylamide-induced neuropathy.

The peripheral neuropathy produced by acrylamide resembles in some respects that produced by methyl butyl ketone and *n*-hexane. In man it affects both sensory and motor nerve fibres and appears to be reversible, although as one would expect, severely affected individuals take longer to recover. A similar type of lesion was produced in the peripheral nerves of experimental animals, but carefully conducted behavioural studies did not reveal effects that could be attributed unequivocally to the nervous system alone.

The studies on amino acid incorporation have so far not elucidated the mechanism of action of acrylamide, despite claims in earlier reports. If one accepts the changes in leucine incorporation in the spinal cord as a reflection of the neural damage produced by acrylamide it would be interesting to learn whether such changes occur at levels below those at which histological damage is apparent. Such comparative experiments would yield information of considerable value to the toxicologist.

[M. A. Thompson—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

PRESERVATIVES

3265. Mutagenicity of cyclic nitrosamines

Stoltz, D. R. & Sen, N. P. (1977). Mutagenicity of five cyclic *N*-nitrosamines: Assay with *Salmonella typhimurium*. *J. natn. Cancer Inst.* **58**, 393.

The cyclic *N*-nitrosamines, nitrosopiperidine (I) and nitrosopyrrolidine (II), are both potent carcinogens (*Cited in F.C.T.* 1974, **12**, 251). However, no carcinogenic effect could be detected in rats from either nitrosoproline (III), which has a similar structure to nitrosopyrrolidine with the addition of a carboxyl group next to the nitroso group, or from its hydroxylated derivative, nitrosohydroxyproline (IV) (Nixon *et al. Fd Cosmet. Toxicol.* 1976, **14**, 133). Rat-liver microsomes hydroxylated I to nitroso-4-piperidinol (Rayman *et al. Biochem. Pharmac.* 1975, **24**, 621) which was found to be carcinogenic (Lijinsky & Taylor, *J. natn. Cancer Inst.* 1975, **55**, 705). This suggested that nitroso-4-piperidinol might be a proximate carcinogenic form of I. A similar hydroxylated metabolite, nitroso-3-pyrrolidinol (V) was identified in the urine of rats given II (Kruger & Bertram, *Z. Krebsforsch.* 1975, **83**, 255) but its carcinogenicity has not yet been investigated. However, both I and II were mutagenic *in vitro* in the presence of a metabolic activation system (McCann *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 5135). In the present study, the mutagenicity of these cyclic nitrosamines both with and without metabolic activation has been further explored.

When compounds I, II, III, IV and V were subjected to an Ames test (using *Salmonella typhimurium* TA1535), only V proved to be mutagenic in the absence of metabolic activation. However, when fortified liver homogenate from rats pretreated with a polychlorinated biphenyl was added to the test mixture, compounds I, II and V all showed evidence of mutagenicity. The findings thus support the hypothesis that hydroxylation of the ring may play a role in the metabolic activation of cyclic nitrosamines to the ultimate carcinogenic form but do not preclude the possibility that other metabolites are more active carcinogens. The mutagenic potential of I, II, III and IV paralleled their previously demonstrated carcinogenic potential *in vivo*, and allowed the prediction that V would show carcinogenic activity.

BLEACHING AND MATURING AGENTS

3266. The effects of chlorinated cake flour

Cunningham, H. M., Lawrence, G. A. & Tryphonas, L. (1977). Toxic effects of chlorinated cake flour in rats. *J. Toxicol. envir. Hlth* **2**, 1161.

To improve its cake-baking qualities, flour is frequently chlorinated at levels of up to 0·25%. The process results in the formation of chlorinated fatty acids and triglycerides, which are absorbed from the gut and deposited in the tissues to a much lesser extent than their unchlorinated counterparts (Cunningham & Lawrence. *Fd Cosmet. Toxicol.* 1977, **15**, 101). Chlorinated compounds in water-soluble extracts of flour or gluten are readily absorbed and excreted, and do not accumulate in the body (*idem, ibid* 1977, **15**, 105). In an early study, rats fed for four generations on chlorinated flour lipids showed no adverse effects at a level equivalent to a diet consisting entirely of flour, although at five times this level there were adverse effects on lactation in females and coat texture in males (*Cited in F.C.T.* 1964, **2**, 496).

In the present study, rats were fed for 2 wk on a diet containing 87% unbleached wheat flour, chlorinated at levels of 0, 0·2 or 1·0%. Body-weight gain was significantly reduced at both levels of chlorination, by 20·7 and 85·2% respectively, while relative liver weight was increased by 16·7 and 25·3%. At the higher level, relative kidney and brain weights were also greater but relative heart weight was unaffected. Similar results were obtained when the extracted lipids from flour chlorinated at the same levels were fed, although the reduction in weight gain at the 1% level of chlorination was far less severe. When rats were fed a chow diet containing 10% wheat gluten chlorinated at levels of 2·0 or 5·8%, growth rate was again reduced and liver weight increased, and at the higher level there was also a significant increase in liver lipids. In a fourth experiment in which rats fed a chow diet containing 0·2% chlorine as chlorinated flour lipids were compared with pair-fed controls, chlorination was found to increase the absolute weight of the liver by 40%, the kidney by 20% and the heart by 10%. There was also a 3·5% decrease in carcass weight and a slight increase in liver content of dry matter, but liver-lipid levels were unaffected.

In rats fed chlorinated wheat flour or lipids extracted from it, the hepatocytes contained irregularly shaped vacuoles that stained with PAS diastase, suggesting the accumulation of glycogen (as might be expected from a high carbohydrate diet). However,

there was no lipid accumulation in the vacuoles. Liver changes in rats fed chlorinated gluten suggested proliferation of the smooth endoplasmic reticulum, which like the increase in dry matter in the fourth experiment was considered indicative of enzyme induction.

Studies with ^{36}Cl showed that in flour chlorinated at 0·2 and 1% the lipid fraction contained 45 and 27% of the chlorine respectively, the remainder being found in the flour residue. The 0·2% level did not affect the amino acid content of the flour, but at 1% the levels of methionine and tyrosine were appreciably reduced.

THE CHEMICAL ENVIRONMENT

3267. Vinyl chloride and the gene

Szentesi, I., Hornyák, É., Ungváry, G., Czeizel, A., Bognár, Z. & Timar, M. (1976). High rate of chromosomal aberration in PVC workers. *Mutation Res.* **37**, 313.

Acro-osteolysis, Raynaud's syndrome and angiosarcoma of the liver are well-known consequences of industrial exposure to high levels of vinyl chloride (*Cited in F.C.T.* 1976, **14**, 347 & 498). Although the *in vivo* mutagenic properties of the monomer are less well established, a number of recent investigations have demonstrated chromosomal aberrations in workers exposed to vinyl chloride (e.g. Ducatman *et al. Mutation Res.* 1975, **31**, 163). Further evidence of the mutagenic activity of vinyl chloride in man is provided by the present study.

Chromosomal analyses of the peripheral blood were performed on a group of 45 workers in the PVC industry who were exposed to vinyl chloride, a control group of 44 individuals engaged in other plants but not directly exposed to chemicals, and a second control group of 49 subjects with no occupational exposure to chemicals. The rate of numerical chromosome aberrations did not differ significantly between PVC workers and controls but the frequency of chromatid-type and unstable chromosome-type aberrations was significantly higher ($P < 0·001$) in the exposed than in either of the two control groups. Five of the 12 men who had been exposed to vinyl chloride for at least 12 yr showed abnormal incidences of both chromatid- and unstable chromosome-type aberrations. In addition, there was one such case among those with 6–7 yr plant experience and another with 10–11 yr experience. However, no cases were found among the 21 PVC workers with 1–5 yr plant experience.

3268. Nasty vinyl chloride associates?

Rannug, U. & Ramel, C. (1977). Mutagenicity of waste products from vinyl chloride industries. *J. Toxicol. envir. Hlth* **2**, 1019.

While the industrial practices associated with the polymerization of vinyl chloride have been the subject of thorough examination, the manufacture of the monomer itself has been given much less attention. The synthesis of vinyl chloride from acetylene or ethylene results in the formation of a by-product, ethylene dichloride tar (EDG-tar), a complex mixture of ethylene dichloride and other chlorinated aliphatic hydrocarbons. This tar has been shown to be toxic to fish and bacteria. The present study reports on its mutagenic activity *in vitro*.

Extracts of EDC-tar in ethanol, dimethyl sulphoxide (DMSO) or 10% Tween 80 (in 0·9% saline solution) were incubated, at levels of 100, 300, 600 or 900 μg per plate, with strain TA1535 of *Salmonella typhimurium*. Methylmethane sulphonate and 2-aminoanthracene were used as positive controls. EDC-tar was both toxic and mutagenic in all three solvents. At the highest concentration in ethanol and DMSO solutions the survival of the bacteria was reduced by 40% and the incidence of mutation was about five times the control value. The Tween emulsion killed 30% of the bacteria and produced a tenfold increase in mutation rate. Similarly, the extracts were all mutagenic and toxic when incubated with the bacteria in the presence of a microsomal fraction obtained from rat liver. However, under these conditions the Tween emulsion was more toxic than the other two EDC-tar solutions, killing 80% of the bacteria at the highest concentration of extract compared with a 50% reduction in survival for both the ethanol and DMSO preparations. The microsomes increased the mutagenicity of all three systems by a factor of two or three and this mutagenic activity was still demonstrable at the lowest dose of 100 μg per plate, a dose at which there was little reduction in survival of the bacteria. Again the Tween emulsion was unusual in that the observed mutagenicity varied only slightly from the lowest to the highest dose of the tar, indicating that a plateau was reached just above the lowest dose. The authors attribute this to possible inhibition of the mutagen-metabolizing system by the Tween 80. When NADPH was omitted, both the potential of the microsomal preparation to enhance the mutagenicity and the toxic activity of the extract were significantly reduced, suggesting that the mixed-function oxidases were involved in the activation step.

Both ethylene dichloride and vinyl chloride have been shown to be mutagenic in the Ames system. However the authors were satisfied that at the very low concentrations at which the compounds were present in the tar, neither could have contributed significantly to the observed mutagenicity and other direct and indirect mutagens must have been present.

NATURAL PRODUCTS

3269. Worth knowing your onions?

Baghurst, K. I., Raj, M. J. & Truswell, A. S. (1977). Onions and platelet aggregation. *Lancet* **I**, 101.

A small-scale study of members of the Jain community in India has suggested that the consumption of onions may have some protective function by affecting factors concerned in atherogenesis (*Cited in F.C.T.* 1977, **15**, 493). Animal studies have indicated that onion or garlic tends to increase fibrinolysis in cholesterol-fed rabbits (*ibid* 1976, **14**, 651).

A small-scale experiment is now reported in which four men and five women aged from 23 to 40 yr were given in succession a control low-fat meal of 1000 kcal (4·2 MJ), a high-fat meal of 2000 kcal (8·4 MJ) containing 160 g saturated fat, and the same high-fat meal to which was added 75 g fried onions. These meals were taken at weekly intervals after an overnight fast. Blood specimens taken before the meal and 2·5 hr after it showed that the high-fat meal produced a significantly greater rate of platelet aggregation than the control meal, but the high-fat meal supplemented with onions did not differ significantly from the control in this respect. The extent of platelet aggregation, however, was not significantly different from the control value after a high-fat meal taken with or without onions. Plasma triglyceride concentrations were significantly higher after each of the two high-fat meals than after the control meal. There was a slight indication of an increased fibrinolysis rate after the onion-supplemented meal, but there was such a wide scatter of individual values that this effect could not be regarded as statistically significant. No differences in blood-glucose concentration or thrombin clotting time appeared.

[The scale of this study renders it of limited value. We are left, moreover, with an equivocal impression that onions may or may not help to avert atherosclerosis.]

3270. New light on alcohol withdrawal

Wood, J. & Laverty, R. (1976). Alcohol withdrawal syndrome following prolonged *t*-butanol administration to rats. *Proc. Univ. Otago med. Sch.* **54**, 86.

The place of acetaldehyde in the metabolic system dealing with ethanol in chronic alcoholics and others is uncertain (*Cited in F.C.T.* 1976, **14**, 220). The possibility that acetaldehyde is concerned in the withdrawal syndrome of alcoholics is discounted in this paper by Wood & Laverty.

Rats were fed a liquid diet containing either 90 ml ethanol/litre or 20 ml *tert*-butanol/litre for 21 days. When administration of the respective alcohols ceased, withdrawal signs appeared, after 2–4 hr in the case of ethanol and after 4–6 hr in the case of *tert*-butanol. Reactions to *tert*-butanol withdrawal were similar in nature to those seen after ethanol withdrawal but were more severe, four of five animals showing forepaw convulsions and all five suffering audiogenic convulsions in response to rattling keys. Three animals died after *tert*-butanol was withdrawn, and all were irritable and aggressive for up to 72 hr. Although there were differences in the delay before onset of withdrawal signs and in their severity, the close resemblance between the withdrawal syndrome of the two alcohols indicates that acetaldehyde, which cannot be formed metabolically from *tert*-butanol, is not responsible for them, in rats at least.

3271. Beer drinking and colorectal cancer

Enstrom, J. E. (1977). Colorectal cancer and beer drinking. *Br. J. Cancer* **35**, 674.

The incidence of colonic cancer is linked with the degree of westernization of the diet, and factors such as a lack of dietary fibre and high intakes of protein and fat have been implicated in its cause (*Cited in F.C.T.* 1976, **14**, 210). Another aetiological factor may be beer drinking, which was strongly correlated with the incidence of both colonic and rectal cancer in a survey of 41 American States (Breslow & Enstrom, *J. natn. Cancer Inst.* 1974, **53**, 631).

The latest study includes 47 of the 51 American States (the other four having been omitted because of insufficient data or heavy alcohol consumption by transient non-residents). The cancer sites involved were: oesophagus, stomach, colon, rectum (colorectal), lung, breast and prostate. When correlation coefficients were calculated between 1960 beer consumption and the 1950–67 average age-adjusted white mortality rates from cancer at various sites, the strongest correlations that emerged ($r = 0{\cdot}81$) were with rectal cancer in males and breast cancer in females. A high correlation was also found with rectal cancer in females, colonic and stomach cancer in both sexes (particularly males) and oesophageal cancer in males. The correlations with rectal, colonic and breast cancer appeared to be even stronger when average annual beer consumption over the years 1941–60 was used in place of the 1960 consumption figures. Beer consumption was also strongly correlated with the ratio of male to female cancer of the rectum, and to a lesser extent of the oesophagus and colon, reflecting the greater consumption of beer by men. When the average annual change in beer consumption between 1945 and 1960 was compared with the average annual changes in death rates for 1950–67, again the strongest correlation emerged with rectal cancer. No allowance was made for a latent period between beer consumption and cancer development (the prohibition of alcohol between 1920 and 1933 being notable in this connexion) as the available data did not permit such analysis.

The only other dietary factor which was as highly correlated with colonic and rectal cancer mortality rates as beer consumption was the *per capita* annual consumption of total absolute alcohol. Lesser correlations were demonstrated with the *per capita* consumption of spirits, fluid milk, cigarettes and wine, but the correlation with beef and fat intakes was very low, in conflict with international findings. Colorectal cancer was also correlated with population den-

sity, urbanization and median *per capita* income, the last somewhat puzzling since no socio-economic gradient in this form of cancer has been discerned. The correlation with total alcohol intake, which displays a very large socio-economic gradient, was also puzzling from this point of view. It was concluded that correlations involving gross population data may be an inadequate indicator of aetiology in chronic diseases such as colorectal cancer, which probably involves several factors. Additional well-designed studies, ideally including a prospective study of groups that differ only in their beer-drinking habits, would be necessary to define the precise role of beer drinking in this respect.

3272. Reassurance for blue cheese addicts

Frank, H. K., Orth, R., Ivankovic, S., Kuhlmann, M. & Schmähl, D. (1977). Investigations on carcinogenic effects of *Penicillium caseicolum* and *P. roqueforti* in rats. *Experientia* **33**, 515.

Some strains of Penicillium used in cheese manufacture, including *P. roqueforti* and *P. camemberti*, have been shown to be capable of toxin production when cultured in a synthetic medium (Lafont *et al. Fd Cosmet. Toxicol.* 1976, **14**, 137). Moreover, an increased incidence of tumours and leukaemia was found in one study when *P. camemberti* var. *candidum* III C 3 was given by gavage or sc injection to rats, although as one tumour was detected in the control group this was of questionable significance (Gibel *et al. Arch. Geschwulstforsch.* 1971, **38**, 1). The possibility that moulds used in camembert and blue cheese manufacture may be carcinogenic has now been further investigated.

Three groups of 60 rats were fed on 5 days weekly for life with one of two types of camembert or one commercial blue cheese, while a fourth group (80 rats) received five types of camembert or brie in weekly rotation. One of the commercial starters used to make the camembert was *P. camemberti* var. *candidum* III C 3. Other groups were treated once weekly by gavage for life or sc for 52 wk with 2·5 ml of a suspension of mycelium from this strain of *P. camemberti*, or one of three other strains used to manufacture the camembert or blue cheese, which had been cultured on wort agar in saline. The mean total dose of mycelium was in the range 4·10–6·23 by gavage, or 2·63–2·94 g by sc injection, while total cheese consumption was in the range 8·0–12·7 kg.

Weight gain and gross and histological findings in all test groups showed no significant differences from controls, and survival was generally longer than in control groups, in which some animals died from an intermittent virus infection. A higher tumour incidence was found in a number of test groups, notably in those given *P. camemberti* var. *candidum* III C 3 by gavage or sc injection. However, mean survival in these groups was 28 and 27 months respectively, with mean tumour induction times of 25 and 24 months, whereas controls survived only 17 months on average and developed tumours after an average of only 11 months. It thus appeared that the higher tumour incidence could be attributed entirely to the longer lifespan of the test animals, and was not indicative of carcinogenicity either in the cheeses or their starters.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3273. Good news for hair-dye users

Kinlen, L. J., Harris, R., Garrod, A. & Rodriguez, K. (1977). Use of hair dyes by patients with breast cancer: a case-control study. *Br. med. J.* **2**, 366.

Some 89% of oxidative hair-dye formulations available in the United States, and 12 of their 18 components, were found to be mutagenic in an Ames test (*Cited in F.C.T.* 1976, **14**, 354). Similar evidence of mutagenicity was obtained with 7 of 11 hair colorants available in the UK, and there were also preliminary indications of an increase in malignant lymphomas in mice when some of these formulations were applied to the skin (Searle *et al. Nature, Lond.* 1975, **255**, 506). Furthermore, 2,4-diaminotoluene, until recently used in hair dyes, has produced hepatocellular carcinomas when fed in a semi-synthetic diet to rats (*Cited in F.C.T.* 1969, **7**, 700). However, three long-term skin-painting tests, in which hair-dye formulations or their individual ingredients have been applied up to twice weekly to rats or mice, have revealed no evidence of carcinogenicity (*ibid* 1973, **11**, 641; Burnett *et al. Fd Cosmet. Toxicol.* 1975, **13**, 353; Giles *et al. J. Toxic. envir. Hlth* 1976, **1**, 539).

There has been little indication that hair dyes may cause cancer in humans apart from a report that, of 100 New York women with breast cancer, 87 had used hair colourings for more than 5 yr (Shafer & Shafer, *N.Y. St. J. Med.* 1976, **76**, 394). Only 26% of women of the same age without breast cancer were regular users, but whether these control subjects were matched in respects other than age was not stated. In the UK, an excess of breast cancer (but of no other listed site of cancer) in single women hairdressers was recorded in the period 1959–63 (Registrar General's Decennial Supplement, England and Wales 1961, Occupational Mortality Tables; HMSO, London, 1971), and a similar but smaller increase occurred in 1949–53. Prompted by these findings, a survey of hair-dye use by breast cancer patients has now been undertaken in the Oxford region.

Of 191 women with breast cancer interviewed in 1975–76, 37 (19·4%) had used semi-permanent hair dyes, 33 (17·3%) had used permanent dyes and 15 (7·9%) had used permanent dye and bleach preparations. When these women were compared with 561 controls without breast cancer, matched for age, marital status and social class, no significant difference

was found in the proportion who had used hair dyes in the different categories (20·0, 16·4 and 7·1% of controls respectively). In addition, there was no appreciable difference between the two groups in the duration of hair-dye use, the frequency of application, or the use of named brands containing diamines. Only 12 hairdressers were involved in the survey, but the proportion was no higher among the breast cancer patients (two) than among the controls (ten). Hair-dye use was found to be positively correlated only with smoking in women aged over 50 yr, and with youth (age 15–19 yr) at first pregnancy. It was calculated that there would have been a 98% chance of detecting a doubling of breast cancer risk if the latent interval was 4–5 yr after hair-dye use and an 85% chance 9–10 yr after use. However, a longer latent period before the onset of breast cancer would not have been detected in this study, as only five patients and 12 controls had used hair dyes for more than 14–15 yr.

TOXICOLOGY

3274. PBB gets the bird

Ringer, R. K. & Polin, D. (1977). The biological effects of polybrominated biphenyls in avian species. *Fedn Proc. Fedn Am. Socs exp. Biol.* **36**, 1894.

As a result of the accidental contamination of cattle-feed in Michigan by polybrominated biphenyl (PBB), many investigations into its biological and toxicological effects on domestic animals and wildlife have been initiated. In mammalian species the effects of PBB appear to be particularly marked in the liver (*Cited in F.C.T.* 1978, **16**, 74). The study cited above reviews the data on PBB toxicity in birds.

Continuous exposure of adult chickens to dietary levels of PBB at 125 ppm resulted in slight inanition. Levels of PBB below this had no effect on food intake, whereas at 625 ppm immediate refusal of food was observed. Japanese quail appeared to be as responsive as chickens; levels of 100 ppm or less had no effect on growth or feed intake, although at 500 ppm or more food was refused and death from starvation resulted. Egg production in chickens was significantly reduced at 45 ppm and above. However, even at a level as high as 3125 ppm, egg production was back to normal within 6 wk after PBB withdrawal. Although 25 ppm PBB in feed for 5 wk did not affect egg production, hens produced fewer eggs when given the compound for 8 wk at 20 ppm. At a dose-level of 625 ppm, PBB caused egg production to cease within days after its introduction into feed. In comparison, PBB reduced egg production from 68 to 17% in Japanese quail fed 100 ppm PBB, but had no effect at 10 or 20 ppm for 9 wk of treatment. None of the eggs hatched when quail were fed at the 100 ppm level. With chickens, this feature was observed at levels between 64 and 90 ppm, and a no-effect level of 20–30 ppm was recorded.

Subcutaneous oedema of the neck and shoulders were common abnormalities of hatched chicks from PBB-fed hens although no characteristic teratogenicity was evident. Those offspring that hatched were less viable during their first 3 wk of life but PBB did not produce thinner egg shells or lower egg weight.

A linear relationship was observed between dietary level of PBB and its egg residue for both Japanese quail and chickens. The ratio of the PBB level in eggs to dietary level was found to be 1·3–1·5:1·0 and the biological half-life of PBB in eggs was calculated to be 17 days. Tissue residues tended to be higher in males than females and it is suspected that egg production is a major excretory route for PBB. The highest levels of PBB residues were found in adipose tissue.

PBB has also been shown to affect the liver, thyroid, testes, comb, heart, spleen, bursa of Fabricius and blood of avian species. Hepatic enlargement was evident in chickens in pair-feeding studies and the liver weight of male Japanese quail was increased linearly as a percentage of body weight, at feed levels of 10–100 ppm PBB. Hepatic porphyria and microsomal enzyme induction also resulted from PBB administration. The sizes of the spleen, bursa and comb were reduced but that of the thyroid was increased after PBB feeding. As with the PCBs, hydropericardium and general oedema are also characteristic toxic effects. Other side effects include depressed haematocrit and haemoglobin values in chickens.

It is concluded that although PBB has many adverse effects in chickens it is generally slightly less toxic than PCB.

THE FOURTH INTERNATIONAL CONGRESS OF PESTICIDE CHEMISTRY (IUPAC) ZURICH JULY 24th–28th 1978

"The 4th International Congress of Pesticide Chemistry will be held under the sponsorship of the International Union of Pure and Applied Chemistry (IUPAC) in Zurich, Switzerland, on 24–28 July 1978. The Scientific programme will consist of symposia, workshops and discussion (poster) sessions and will be divided into seven sections (main topics) including:

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Congress language is English.

For further information contact: M Spindler, Congress Secretariat, 4th International Congress of Pesticide Chemistry, P.O. Box 182, CH-4013 Basle/Switzerland".

NEW IUPAC PUBLICATIONS

ETHYLENETHIOUREA

P.C. KEARNEY, *US Department of Agriculture, Beltsville, Maryland*

International Union of Pure and Applied Chemistry: Pesticide Terminal Residue Commission, Applied Chemistry Division. A special report on the occurrence of Ethylenethiourea as a terminal residue resulting from agricultural use of Ethylenebisdithiocarbamate.

Ethylenethiourea is a well known and fairly stable compound. It is employed commercially or industrially as an accelerator in the production of synthetic rubber. When administered under various toxicological experimental conditions, ETU has been shown to have caused, or in some cases suspected to have caused various pathological effects. These, including goiterogenic, tumorogenic and teratological effects, have raised some questions regarding the possible harmful effects as terminal residues of the relevant dithiocarbamate fungicides.

Contents: 1. Analytical Methodology. 2. Chemistry of Ethylenethiourea (ETU). 3. Dynamics and transformation of ETU residues. 4. Processing and cooking effects on EBDC'S. Conclusions. Recommendations.

Of interest to: Graduate and research workers in organic chemistry, farmers, agricultural advisers and pest control specialists. Processed food manufacturers.

ISBN 0 08 022026 6 f US$ **6.00** **£3.30**
14pp 273 x 188mm August 1977

Previously published in **Pure & Applied Chemistry,** Volume 49 No. 5, and supplied to subscribers as part of their subscription.

ADVANCES IN SMOKING OF FOODS

A RUTKOWSKI, *Agricultural University of Warsaw, Poland*

This international symposium was sponsored by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Food Sciences and Technology (IUFoST), Warsaw, 8 - 10 September, 1976.

Although the smoking or smoke flavouring of foods is an age-old process, many changes have occurred in recent years. Four general factors have influenced these changes, and/or supplied the need for change, namely, (1) the desire for the better control of the flavour and colour of smoked foods, (2) the concern for possible carcinogenesis by components in wood smoke, (3) the need to reduce smoke effluent and (4) the constant need for better plant design and faster output.

Contents: The phenomena of quality in the smoke curing process, TILGNER. Physical and chemical processes involved in the production and application of smoke, RUSZ and MILER. Analysis of smoke and smoked foods, HAMM. Contribution of smoke compounds to sensory, bacteriostatic and anti-oxidative effects in smoked foods, BARYLKO-PIKIELNA. Some facts and legislation concerning polycyclic aromatic hydrocarbons in smoked foods, WALKER. Novel concepts in technology and design of machinery for production and application of smoke in the food industry, HOLLENBECK.

Of interest to: Manufacturers and food technologists, bacteriologists, physicochemists and organic chemists.

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MYCOTOXINS IN FOODSTUFFS - 3

M. JEMMALI, *INSERM–INRA–16, rue Nicolas Fortin 75013, Paris*

The Third International Symposium on Mycotoxins in Foodstuffs, Paris 16 - 18, 1978 was sponsored by the International Union of Pure and Applied Chemistry (IUPAC), The French Committee for Chemistry and the French Committee for Sciences of Nutrition.

The emphasis of the Symposium was on control measures for mycotoxins in foodstuffs and on the toxicological aspects. The important subject of decontamination was one of the key features of the Symposium.

Contents: Food mycotoxins survey and monitoring programs, CAMPBELL. Sampling granular foodstuffs for aflatoxin, WHITAKER. Mycotoxin tolerances in food-stuffs, KROGH. Mode d'action des mycotoxines, MOULE. Mode of action of Trichothecenes, UENO. Mode of action and human health aspects of aflatoxin carcinogenesis, BUTLER and NEAL. Short-term tests and mycotoxins, MALAVEILLE and BARTSCH. Review of prevention, elimination and detoxification of aflatoxine, GOLDBLATT and DOLLEAR. The mycotoxins and human health hazards, LINSELL. Metabolism of aflatoxin and other mycotoxins in relation to their toxicity and the accumulation of residues in animal tissues, PATTERSON. Some new mycotoxins, STEYN.

Of interest to: Research workers from university, industry and government institutions dealing with applied biology, microbiology, contamination of raw materials and phytopathology.

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[*Contents continued*]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

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e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation—I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

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