Proc. Nutr. Soc. (1981), **40**, 67

The influence of the gut microflora on food toxicity

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The impressive variety of reactions that can be performed by the gut flora on both exogenous and endogenously-produced compounds justifies its status as a major site of metabolism, comparable to the liver (Drasar *et al.* 1970; Scheline, 1973; Walker, 1973; Drasar & Hill, 1974). Furthermore, one might expect that the capacity of the flora to adapt to metabolic pressure by altering its component organisms as well as by induction of enzymes, would exceed that of the liver. Surprisingly, however, although hepatic enzyme induction has been extensively studied, metabolic adaptation by the gut flora remains a largely unproven theory, with the notable exception of cyclamate metabolism (Drasar *et al.* 1972).

If the metabolic activity of the intestinal bacteria is compared to that of the liver it is apparent that the bacterial biotransformations both complement and antagonize hepatic metabolism. Whereas reactions in the liver are mainly oxidative in character and syntheses are common (for example, production of glucuronides and sulphate conjugates) the lack of molecular oxygen in the intestinal lumen limits the number of oxidations that can take place and hence reductive and degradative metabolism predominates. Synthesis by the gut flora is rare with the notable exceptions of nitrosamine formation (Sander, 1968; Hawksworth & Hill, 1971) and alkylation of metals (Rowland *et al.* 1977*a*). The reactions of the intestinal bacteria therefore complement those of the liver and the net result is an expansion of the range of possible biotransformations to which an ingested compound can be subjected.

The gut flora antagonizes hepatic metabolism by hydrolysing the biliary conjugates (such as glucuronides, sulphates and amides) synthesized by the liver in an attempt to excrete toxic non-polar drugs and anutrients. Hydrolysis of the conjugates can lead to an enterohepatic circulation (Williams *et al.* 1965) which not only delays excretion due to reabsorption of the parent compound into the portal system, but also permits further metabolism of the substance due to its subsequent passage through the liver.

Some of the toxicological consequences of gut flora metabolism have been discussed by Drasar *et al.* (1970), Scheline (1972, 1973) and Walker (1973) and are summarized in Table 1. In the present paper, rather than giving a series of brief examples to illustrate the points in Table 1, I shall confine the discussion to two classes of compound (azo dyes and heavy metals) which have been extensively studied with respect to their bacterial metabolism.

0029-6651/81/4013-4603 $01.00 © 1981 The Nutrition Society

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Table 1. *Toxicological consequences of foreign compound metabolism by the gut flora*

1. Formation of toxic, carcinogenic or mutagenic metabolites
2. Detoxication
3. Enterohepatic circulation
4. Differences or changes in metabolism and toxic effects due to:
   (a) Species, strain and individual differences in gut flora composition
   (b) Species differences in distribution of flora
   (c) Dietary modification of flora
   (d) Metabolic adaptation
   (e) Effects of disease and gastrointestinal disorders on composition and distribution of flora

Azo dyes

Metabolism

Azo compounds are extensively used as colouring agents in food, drink, drugs and cosmetics and consequently their metabolism and toxic activity have been studied in detail (see reviews by Walker, 1970, and Drake, 1975). The dyes are derivatives of aromatic amines and the presence of various polar and non-polar substituents (-CH_3,-SO_3Na,-OH) imparts different degrees of water-solubility to the dyes. Although azo reductase is present in both the liver and the intestinal flora, the relative unimportance of the former source of the enzyme in the metabolism of the water-soluble azo dyes Amaranth, Ponceau SX and Sunset Yellow FCF was established by Radomski & Mellinger (1962) who studied the absorption and excretion of the dyes after oral and intravenous dosing and after administration to rats treated with antibiotics to depress microbial activity. This and other studies (Walker, 1970) have shown that parenteral administration of dyes results in excretion of unreduced dye in urine and bile whereas after oral administration reduction of the azo group and excretion of amine products occurs.

The poor absorption of the water-soluble sulphonated azo dyes from the gut increases the likelihood of their reduction by the gut flora, while it would appear that lipid soluble azo compounds, which are absorbed more readily, are metabolized by both hepatic and intestinal azo reductase (Gingell *et al.* 1969; Walker, 1970).

The metabolism in vitro of a variety of azo dyes by suspensions of gut contents has been demonstrated (Scheline & Longberg, 1965; Fore *et al.* 1967; Roxon *et al.* 1967) and although all dyes examined were reduced, the rate of metabolism varied considerably. For example, in 4 h 85·5% of Amaranth, but only 37·5% of Tartrazine was reduced (Roxon *et al.* 1967). In similar studies Linnecar (1974) concluded that the polarity of the dye influenced its rate of metabolism since the water-soluble azo dyes such as Amaranth, Ponceau 4R, Sunset Yellow were rapidly metabolized by gut bacteria, whereas dyes which gave rise to non-polar metabolites such as Orange G, Orange RN and Red 10B were less readily reduced.

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The ability to reduce azo dyes is widely distributed amongst the intestinal bacteria including *Streptococcus faecalis* (Scheline *et al.* 1970), *Proteus* (Roxon *et al.* 1966, Dubin & Wright, 1975), *Bacteroides fragilis* ss. *thetaiotaomicron, Eubacterium biforme, Peptostreptococcus* spp. and *Fusobacterium* spp. (Chung *et al.* 1978).

Formation of toxic metabolites of azo dyes by bacterial azo reductase

A classic example of the generation of toxic products by the gut flora is afforded by the food dye Brown FK, which is a complex mixture of at least six *p*-sulphophenylazo derivatives of 1,3-diaminobenzene and 2,4-diaminotoluene (Fore & Walker, 1967). The major importance of the gut flora in the metabolism of the dye was established by showing that in the presence of rat gut contents, Brown FK underwent azo reduction with the formation of sulphanilic acid and a phenazine material, the latter thought to be derived from the condensation of polyamines such as 2,4,5-triaminotoluene and 1,2,4-triaminobenzene which are unstable on exposure to air (Fore *et al.* 1967; Walker *et al.* 1970). The administration of the dye intragastrically to rats, pigs, rabbits and guinea pigs resulted in the excretion of substantial amounts of sulphanilic acid in the urine and faeces whereas after intraperitoneal dosing to rats the dye was mainly excreted in the urine unchanged or as metabolites with an intact azo group (Fore *et al.* 1967). The phenazine material was found in caecal contents of rats dosed orally with Brown FK.

The different metabolic fate of Brown FK when given orally or parenterally was reflected in the toxic response of rats given the dye by the two routes. Rats given repeated oral doses of Brown FK of 0·5–2 g/kg per d suffered from precipitate weight loss and vacuolar myopathy of heart and skeletal muscle (Grasso *et al.* 1968). In contrast, after intraperitoneal administration of equivalent doses no muscular lesions were seen, and furthermore, the incidence of the lesions, particularly the cardiac damage, after oral dosing, could be reduced by pre-treatment of the animals with antibiotics. Clearly, the intestinal microflora is implicated in the production of the toxic metabolites.

Two of the components of the Brown FK dye mixture namely 2,4-diamino-5-(*p*-sulphophenylazo)toluene and 1,3-diamino-4-(*p*-sulphenylazo)benzene were found to reproduce the toxic effects of the dye when given orally (Grasso *et al.* 1968) and the amines derived from these compounds by azo-reduction in vitro, namely 2,4-5-triaminotoluene and 1,2,4-triaminobenzene caused cardiac and muscular lesions in rats after intravenous injection (Walker *et al.* 1970).

Although the pathological lesions induced in rats by Brown FK could be reproduced in rabbits and guinea pigs, mice were not susceptible. However, cardiac and muscular lesions were apparent in mice after intravenous injection of the two toxic polyamines, suggesting that differences in intestinal microflora are responsible for the differential susceptibility of rats and mice to oral administration of Brown FK (Walker *et al.* 1970).

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Formation of carcinogenic metabolites of azo dyes by bacterial azo reductase

A high incidence of bladder cancer was noted in Japanese workers using benzidine-derived azo dyes, many of which are used in textile and rubber industries (Yoshida & Miyakawa, 1973). The dyes were found to be metabolized by intestinal bacteria with the production of benzidine, a known human bladder carcinogen. Furthermore, in Rhesus monkeys fed the dyes free benzidine was found in the urine (Rinde & Troll, 1975).

Certain azo dyes containing methylated aniline moities have been shown to possess carcinogenic activity or produce hyperplastic lesions, notably Ponceau 3R and Ponceau MX (Grice *et al.* 1961; Grasso *et al.* 1969; Mannell, 1964). Both dyes are reduced rapidly by intestinal bacteria (Hartman *et al.* 1979; I. R. Rowland, unpublished observation) with various trimethyl and dimethylanilines being the likely reduction products. Of these amines, 2,4,5-trimethylaniline (from Ponceau 3R) has been shown to be carcinogenic to the liver of rats (Morris & Wagner, 1964) and 2,4,5-trimethylaniline and 2,4-dimethylaniline to be mutagenic in bacteria (see below).

The dye Orange II is azo-reduced by many intestinal bacteria (Chung *et al.* 1978) and one of the products, 1-amino-2-naphthol, produced bladder tumours after implantation in the mouse bladder (Bonser *et al.* 1963).

Activation of azo dyes to mutagens by bacterial azo reductase

The advent of the very rapid in vitro assays for mutagenicity and carcinogenicity has enabled extensive testing of food colourings (Brown *et al.* 1978, 1979; Garner & Nutman, 1977; Haveland-Smith & Coombes, 1980*a*,*b*). However, it became clear that the standard in vitro methods of testing for mutagenicity were inadequate since the metabolizing system used in the tests (a rat liver homogenate) contains only hepatic azo reductase which may not be very active under the aerobic conditions used in most in vitro mutagenicity tests. Consequently, more recent studies of the mutagenicity of food dyes have utilized known azo reduction products (Garner & Nutman, 1977), chemical reduction of the dyes with dithionite (Brown *et al.* 1978) or bacterial metabolizing systems (Hartman *et al.* 1978, 1979) although in the case of the *Salmonella*/microsome mutagenicity test, the bacterial test organisms themselves may reduce the dyes (Garner & Nutman, 1977; Haveland-Smith & Coombes, 1980*a*).

Using a cell free extract of an anaerobic bacterium (*Fusobacterium* sp.) Hartman *et al.* (1979) showed that the dye Trypan blue was converted by azo reduction to the mutagenic and carcinogenic substance *o*-tolidine.

Brown *et al.* (1978) demonstrated that certain azo dyes such as Sudan IV Acid alizarin and Violet N required chemical reduction and microsomal activation for their mutagenic activity to be revealed. Similarly, 1,2,4-triaminobenzene, a reduction product of the mutagenic dye Chrysoidin was found to be mutagenic after activation by liver (Garner & Nutman, 1977). The dyes Ponceau MX and Ponceau 3R which have been shown to be carcinogenic are not mutagenic *per se*

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nor in the presence of a rat liver homogenate (Hartman *et al.* 1979; I. R. Rowland, unpublished observation). However, the reduction products 2,4,5-trimethylaniline (from Ponceau 3R) and 2,4-dimethylaniline (from Ponceau 3R and Ponceau MX) are both mutagenic in the presence of liver homogenates. Other reduction products of these two dyes, 2,5- and 2,6-dimethylaniline were mutagenic without microsomal activation, but only at high concentrations (I. R. Rowland, unpublished observation).

Reduction in mutagenic activity of azo dyes by bacterial metabolism

Although azo reduction of food dyes can yield mutagenic or promutagenic metabolites, in some cases, the reduction wholly or partly abolishes the mutagenic effects of colours. For example, Red 10B and Red 2G when subjected to anaerobic incubation in vitro with rat caecal contents, lose their genotoxic activity (Haveland-Smith & Coombes, 1980*b*).

Effect of diet on azo reduction

The activity of hepatic and bacterial azo-reductase is increased in the presence of riboflavin (Williams *et al.* 1965) since the latter is a required cofactor for the enzyme (Mueller & Miller, 1950) hence dietary riboflavin can affect the toxicity of compounds which depend on azo reduction for activation or detoxication. For example, the carcinogenicity of Butter Yellow (4-dimethylaminoazobenzene) is decreased by dietary riboflavin (Miller & Miller, 1953).

Dietary changes less specific than an alteration in riboflavin content, have also led to changes in azo reductase levels in the gut flora. Rats fed a diet predominantly consisting of beef had higher levels of azo-reductase in their faecal flora than when they were fed on diets based on grain and vegetables (Goldin & Gorbach, 1976). The changes in enzyme activity occurred over a period of 4–10 d after transfer from the vegetable to the meat diet and roughly corresponded to changes in faecal flora which took place during this time (Weinstein *et al.* 1974).

Biotransformation of toxic metals by the gut flora

The toxicity and biological activity of many metals and metalloids is profoundly influenced by their chemical form, for example, alkyl mercury compounds are absorbed rapidly from the mammalian gut and are much more toxic when given orally than inorganic mercurials and metallic mercury which are poorly absorbed from the gut (Friberg & Vostal, 1972). Contamination of food by heavy metals and metalloids is common, especially with mercury, cadmium, arsenic and lead and the metabolism of ingested metals could significantly modify their toxicity. The discovery that micro-organisms in lakes, rivers and soil could biotransform metallic compounds (Jensen & Jernelöv, 1969; Wood, 1974) stimulated interest in the possibility that similar reactions could occur in the mammalian intestinal tract.

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Intestinal bacteria can subject metal compounds to a wide variety of transformations which drastically alter the potential toxicity of the metal and the reactions can be used to illustrate two comparatively rare properties of the gut flora, namely biosynthetic reactions and detoxication.

Synthesis of methylated mercury and arsenic compounds

Incubation of mercuric chloride with suspensions of rat caecal contents or human faeces yields small amounts of the highly toxic methylmercury (Rowland *et al.* 1977*a*; Edwards & McBride, 1975). The inhibitory effect of antibiotics on the reaction suggested that the bacterial flora was involved and this was confirmed by demonstrating methylmercury synthesis by pure cultures of bacteria isolated from the human and rat intestinal tracts (Rowland *et al.* 1975; Rowland *et al.* 1977*a*). In the case of mercury, therefore, methylation by the gut flora increases the toxicity of the metal.

Under similar conditions, rat caecal suspensions can reduce and methylate sodium arsenate (I. R. Rowland and M. J. Davies, unpublished observations). In contrast to the methylation of mercury, however, this leads to detoxication of arsenic since the products of methylation (methylarsonic acid and dimethylarsinic acid) are less toxic than the inorganic forms of arsenic (Vallee *et al.* 1960).

Metabolism of organomercurials

Incubation of methylmercuric chloride with suspensions of rat gut contents results in the conversion of the organomercurial into one or more volatile products over 2 or 3 d (Rowland *et al.* 1977*b*). The bacterial flora was again shown to be responsible for the transformations. One of the major products of the reaction is metallic Hg, which has a very low oral toxicity. Hence the reduction of methylmercury to metallic Hg can be considered a detoxication reaction.

The significance of the bacterial metabolism of methylmercury on the tissue distribution and toxicity of methylmercury in the rat has been assessed by the use of animals treated with antibiotics to suppress the metabolic activity of the gut flora (Rowland *et al.* 1980).

By comparison to conventional animals with an intact gut flora, antibiotic-treated rats given methylmercuric chloride intragastrically had significantly more Hg in their tissues (notably kidney, lung, blood, brain and muscle) and excreted less in the faeces. In addition, the percentage of Hg as methylmercury was higher in kidneys of antibiotic-treated rats than in the conventional rats. It appears, therefore, that metabolism of methylmercury by the gut flora reduces the tissue content of Hg.

After large oral doses of methylmercuric chloride, the incidence and severity of the neurological signs of toxicity and histopathological lesions induced by the organomercurial in rats were lower in the conventional animals than in those given antibiotics, demonstrating the protective effect of the gut flora against methylmercury toxicity (Rowland *et al.* 1980).

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Conclusions

Over the last 10–15 years great progress has been made in assessing the contribution of the gut flora to the metabolism and toxicity of food components and additives. In general, the toxicological consequences of metabolism by intestinal bacteria are detrimental to the host animal, with only a small number of reactions resulting in detoxication.

There are aspects of gut flora metabolism which would benefit from further study. Among the most important of these is the degree of variation of the gut flora (especially in metabolic activity) between animal species and strains. The differential effects of Brown FK in rats and mice, discussed above, exemplifies the dramatic difference in toxicity that can result from interspecies differences in metabolism by the gut flora.

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Printed in Great Britain

https://doi.org/10.1079/PNS19810011 Published online by Cambridge University Press