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REDUCTION BY THE GUT MICROFLORA OF ANIMALS AND MAN

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Reduction by the gut microflora plays an important role in the metabolism and toxicity of a number of drugs and other foreign compounds, particularly those containing nitro groups. The metabolism of such drugs may be modified by a variety of factors which influence the reductive capacity of the microflora including diet. In addition there are numerous differences in the gut microflora between individuals and animal species which makes extrapolation of metabolic and toxicological data from laboratory animals to man extremely difficult.

By comparison to the mammalian liver, in which oxidative metabolism predominates, the gut microflora, by virtue of the low redox potential of the gut lumen, is very active in reductive reactions and its action on a wide variety of substrates has been described [1, 2] (Table 1). Clearly, ingested compounds which are poorly absorbed from the gut will stand the greatest chance of undergoing metabolism by the intestinal flora, although a large number of compounds gain entry to the gut via biliary secretion and so will be also exposed to microbial action. Although the liver possesses reductive enzymes, including nitro- and azoreductases, for many compounds hepatic metabolism appears to play a minor role in their reductive biotransformation.

Wheeler *et al.* [3] demonstrated that germ-free rats converted only 1% of an oral dose of *p*-nitrobenzoic acid to *p*-aminobenzoic acid compared to 25% conversion in conventional microflora animals. These results have been confirmed using antibiotic treated rats and marmosets [4–6]. The metabolism of metronidazole to acetamide and *N*-(2-hydroxyethyl)-oxamic acid, reactions shown to occur in the presence of intestinal bacteria *in vitro*, has been demonstrated in conventional rats but not in germ-free rats [7, 8], and the products of nitro-reduction of chloramphenicol (namely the arylamine and acetylarylamine derivatives), which are major metabolites of the drug found in urine and faeces of conventional rats, comprised less than 7% of the metabolites in germ-free animals [9]. Similarly, the excretion of reduced metabolites of the hepatocarcinogen 2,4-dinitrotoluene by germ-free rats was only 10–20% of that in conventional microflora animals [10]. The gut microflora of man and several experimental animals can reduce a wide variety of azo compounds *in vitro*. *In vivo*, these compounds, particularly the water-soluble azo dyes which are poorly absorbed from the gut, have been shown to be reduced almost exclusively by the gut microflora [11, 12]. The early antibiotic drugs, prontosil rubrum and neoprotosil, which are azo derivatives of sulphonamides, were shown to be activated by microbial azo reductase in the gut [2]. More recently, the drug sulphasalazine (salicylazosulphapyridine), which is commonly used to treat patients with ulcerative colitis, has been found to be almost completely metabolized in the colon and caecum by bacterial azo reductase to yield sulphapyridine and 5-aminosalicylic acid. These are largely absorbed from the colon and undergo further metabolism by mammalian enzymes before excretion [13, 14]. The pathways of metabolism of sulphasalazine appear to be similar in the rat and man where less than 10% of an oral dose is recovered with an intact azo link in faeces and urine [14–16]. When sulphasalazine is fed to germ-free rats little or no reductive metabolites are detected in faeces or urine, the unchanged drug being the main excretion product indicating that mammalian azo reductase plays an insignificant role in the metabolism of the drug [14, 15]. In addition bacteria from the human and rat intestinal tracts can reduce the azo bond of the drug in *in vitro* incubations [14].

It is clear from the above studies that the intestinal microflora is of paramount importance to the reduction of many drugs and foreign compounds, particularly those containing nitro or azo groups.

Table 1. Reduction by the gut flora

1.	—CH=CH— bonds
2.	Azo compounds
3.	Nitro compounds
4.	Nitrate
5.	Nitrite
6.	*N*-oxides, *N*-hydroxy compounds
7.	Carbonyl compounds
8.	Alcohols, phenols
9.	Arsonic acids
10.	Reductive dehalogenation

Table 2. Factors affecting drug metabolism by the gut microflora

1.	Individual variation in gut flora
2.	Species variation in gut flora
3.	Species variation in distribution of flora within the gastrointestinal tract
4.	Gastrointestinal disease
5.	Age
6.	Diet
7.	Antimicrobial drugs and agents affecting gastrointestinal physiology and secretions

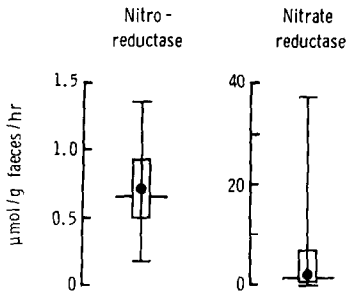

Fig. 1. Distribution of data for reductive enzyme activities in faecal samples from six humans. Values as presented as mean (———), median (●) range (I) and interquartile range (□) for 4 control periods (3 samples per period).

Consequently, it is important to bear in mind the factors which may influence the flora's metabolic capacity. In this respect it should be noted that the gut flora is very susceptible to change and numerous factors may influence the microbial reduction of drugs. The most important of these are listed in Table 2 and some are discussed in more detail below.

INTER-INDIVIDUAL VARIATION

In man the gut flora is extremely complex [17] and displays marked differences between individuals in the same population. This is reflected in wide variations in metabolic activity between individuals (Fig. 1). In a study of reductive reactions in faecal preparations from a group of medical students consuming a normal diet there was a 7-fold range in the rate of *p*-nitrobenzoic acid metabolism between the six individuals.* On the other hand, the enzyme activities for each individual were fairly consistent over a long period of time (5 months).

Several studies have indicated that there are differences in bacterial composition of gut flora between different human populations, e.g. between those of the U.K. and India or Africa [18, 19]. These differences have not always been borne out by subsequent studies [17] and it would be speculative to comment on differences in reductive capacity between such population groups.

DIFFERENCES BETWEEN THE GUT FLORAS OF ANIMALS AND MAN

Because of the extensive use of laboratory animals in the testing of clinical efficiency and safety of drugs, it is pertinent to consider the differences between the gut floras of such animals and man. Although there have been numerous studies of the microbial composition of the floras of animals and man (reviewed by Rowland and Walker [2]), it is difficult to relate these to the metabolic capacity of the flora as a whole and so I shall confine my comments to comparisons of enzymic activity between the various mammalian gut floras. Marked differences have been found in the activities of reductive enzymes associated with the caecal or faecal floras of rats, mice, hamsters, guinea pigs, marmosets and humans (Fig. 2) with no single species exhibiting consistently higher or lower enzyme activities.† Furthermore none of the laboratory animals, including the marmoset, provided a good model of the enzymatic activity associated with the human faecal flora. The results in Fig. 2 imply that certain nitro compounds such as nitrobenzenes and dinitrotoluenes which depend on reduction by the gut flora for their toxic effects [20, 21] would be more potent in rats, mice and hamsters than in humans, who had much lower bacterial nitroreductase activity than the laboratory animals.

This study was performed using animals of one strain only within each species so there may be other strains which show closer similarities to man. More importantly the reductive capacity of the flora can be altered by diet so it may be possible to obtain a

* A. K. Mallett, I. R. Rowland and M. J. G. Farthing, unpublished observation, 1985.

† I. R. Rowland, A. K. Mallett, C. A. Bearne and M. J. G. Farthing, submitted for publication 1985.

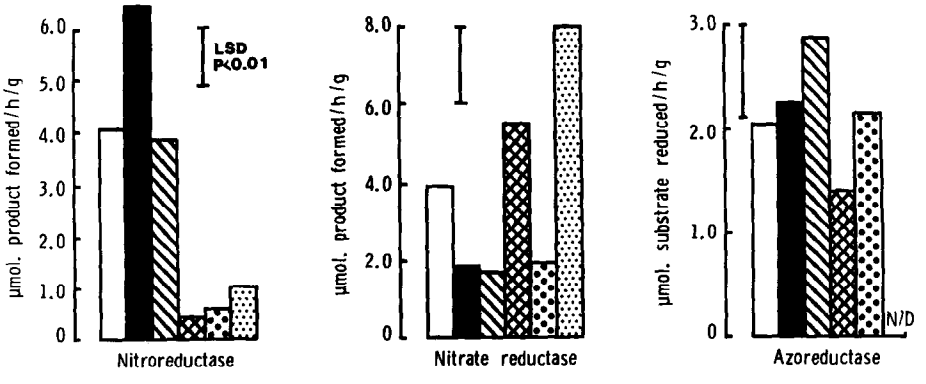

Fig. 2. Enzyme activities of faeces or caecal contents of laboratory animals and man. Suspensions (10% w/v) of caecal contents from rats, mice, hamsters and guinea pigs, or freshly voided faeces from marmosets and humans were prepared in 0.1 M phosphate buffer kpH 7.0 and enzyme activities were measured. The values represent the means of 6 animals and 4 humans and may be compared for statistically significant differences by the appropriate least significant difference value ($P < 0.01$). Key: LSD, least significant difference value; ND, not determined; □ rat; ■ mouse; ▨ hamster; ▩ guinea pig; ⊡ marmoset; ⊞ human.

closer animal model of a human gut flora by an alteration in laboratory animals diet (see below).

Differences in distribution of the gut flora between animals and man

As well as differences in the rates of microbial reductive metabolism between laboratory animals and man there are marked dissimilarities in the distribution of bacteria within the gastrointestinal tracts of different animal species, which have potentially profound consequences for drug metabolism. The most notable differences (which are summarized in Fig. 3) lie in the upper regions of the gut (stomach and duodenum), which in man harbour only a transient flora of 10^1–10^3 organisms per g contents, but which in most laboratory rodents are fairly heavily colonized by a mixed bacterial population of about 10^7–10^8 organisms/g [2]. The reason for these differences may be ascribed to bactericidal action of the strongly acidic gastric juice of the human, whereas the pH of the rat and mouse stomach is more moderate (pH 4–5). The distal regions of the small intestine and the colon of most animal species, including the human, harbour an abundant microflora with numbers of 10^{10}–10^{11} organisms/g often being reached. The differences in gut flora between animal species, summarized in Fig. 3, should be considered in relation to the main sites of absorption of drugs and other foreign compounds namely the duodenum and proximal small intestine [22]. The colonization of stomach and small intestine of the rat and mouse means that a substance has a greater chance of microbial transformation in these animals than in the human, where a drug may be absorbed before reaching the heavily populated regions of the gut. It should be remembered, however, that gastrointestinal disorders, such as hypochlorhydria, which result in bacterial colonization of the stomach and upper small intestine of man are not uncommon and may modify the metabolism of ingested drugs [23, 24].

Age

In human and laboratory animals the gut flora shows a distinct succession of species during early development of the animal and there are numerous differences between the adult and infant gut floras [25, 26]. It is likely therefore that the drug metabolising activity of the flora also exhibits developmental changes but these have received little attention.

Diet

Consistent diet-induced changes in the species composition of the gut microflora have been hard to demonstrate, due in part to the complexity of the bacterial population and to the technical difficulties associated with identifying and enumerating the organisms present [27]. Recently, however, evidence has been accumulating that the metabolic activity of the flora can be radically altered by diet, in particular by those components which escape digestion by mammalian intestinal enzymes, namely dietary fibre.

The major non-fermentable carbohydrate component present in many dietary fibres is cellulose which, when added to a purified rodent diet at 0–40%, caused a marked concentration-dependent decrease in total numbers of micro-organisms [28]. This was accompanied by a decrease in the activities of several microbial enzymes including azo, nitro- and nitrate reductases. The results indicate that cellulose exerts these effects by simply diluting the bacterial population of the large intestine.

Plant cell wall material also contains a number of fermentable components, notably the pectins and hemicelluloses, which have been reported to give dramatic changes in the metabolic activity of the large bowel flora, presumably by providing a source of energy for the bacterial population [29–32]. In a comparision of a number of plant derived hydrocolloids, Mallett *et al.* [32] contrasted the effect of pectin and carrageenan (an algal polysaccharide) on microbial reactions in the gut. When incorporated into a purified rodent diet, at 50 g/kg diet, pectin caused a general increase in azo-, nitro- and nitrate reductase activities although the results were not always statistically significant. The effect on nitrate reductase activity was most marked (Fig. 4). By contrast, dietary carrageenan greatly decreased all the enzyme activities, particularly that of nitroreductase (Fig. 4). The study was extended to other classes of nitro compound including nitrofurantoin,

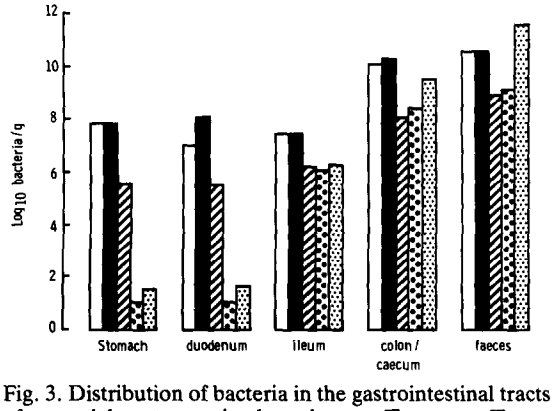

Fig. 3. Distribution of bacteria in the gastrointestinal tracts of some laboratory animals and man: □ mouse; ■ rat; ▨ guinea pig; ▩ rabbit; ▦ man.

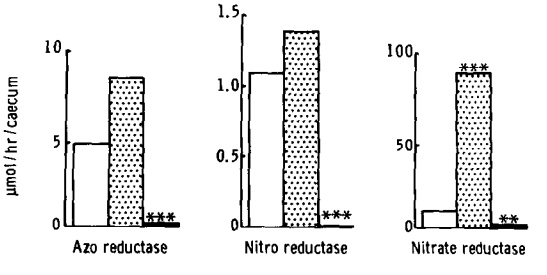

Fig. 4. Effect of dietary pectin and carrageenan on reductive enzymic activities in rat caecal contents. Enzyme activities were measured in suspensions of caecal contents from male Sprague Dawley rats fed, for 4 weeks, a purified fibre free diet (□), or that diet supplemented with 5% (w/w) pectin (□) or carrageenan (■). Values shown are medians for six rats. Those marked with asterisks differ significantly from the control, fibre-free diet (**$P < 0.01$; ***$P < 0.001$, Mann Whitney U Test).

metronidazole and dinitrotoluene [33]. Reduction of all the nitro compounds by the rat caecal flora was significantly decreased by feeding iota carrageenan whereas dietary pectin increased the reduction of metronidazole and *p*-aminobenzoic acid (Fig. 5).

SOME TOXICOLOGICAL IMPLICATIONS OF REDUCTION BY THE GUT FLORA

The importance of the gut flora to the metabolism and genotoxicity of 2,4-dinitrotoluene has been demonstrated by Rickert *et al.* [10] and Mirsalis *et al.* [21]. The amounts of reduced metabolites excreted by germ-free animals were 1/10 to 1/5 those excreted by their conventional microflora counterparts. In addition, hepatic covalent binding of dinitrotoluene metabolites was decreased by about 50% in germ-free animals and the induction of DNA repair (a response to DNA damage) seen in dinitrotoluene-treated conventional animals was absent when germ-free animals were similarly treated.

The gut microflora also plays a crucial role in the induction of methaemoglobinaemia by 1,3-dinitrobenzene. Dose-related increases in methaemoglobin (up to 63% by 50 mg/kg) in blood were apparent when conventional flora Fischer 344 rats were given 1,3-dinitrobenzene. Correspondingly dosed germ-free rats developed only 18% methaemoglobin and no dose-related effects were detected (M. Philbert, personal communication 1985).

Some diet-induced changes in enzymic activity of the gut flora have been shown to result in alterations in susceptibility of the host animal to toxic effects of bacterial reduction products. The most notable examples are the increased susceptibility to nitrate-induced methaemoglobinaemia in rats fed dietary pectin which corresponded with an increase in the activity of nitrate reductase (Fig. 6 [29]) and the increased genotoxicity of 2,6-dinitrotoluene in pectin-fed F344 rats which were shown to exhibit elevated rates of dinitrotoluene metabolism by gut microorganisms [31].

CONCLUSIONS AND SUGGESTIONS FOR ALTERNATIVE METHODOLOGY

It is apparent from the above discussion that the gut microflora plays an important role in the reduction of foreign compounds and that this microbial metabolism may have clinical and toxicological consequences for the host animal. Because there are marked differences between animal species in the distribution of the microflora and its metabolic capacity, the extrapolation to man of data from metabolic, pharmacological or toxicological studies in laboratory animals should be performed with caution.

It is important to bear in mind also the influence of dietary components on the metabolic activity of the flora and the potentially confounding effects of the variations in human food intake. There is a need,

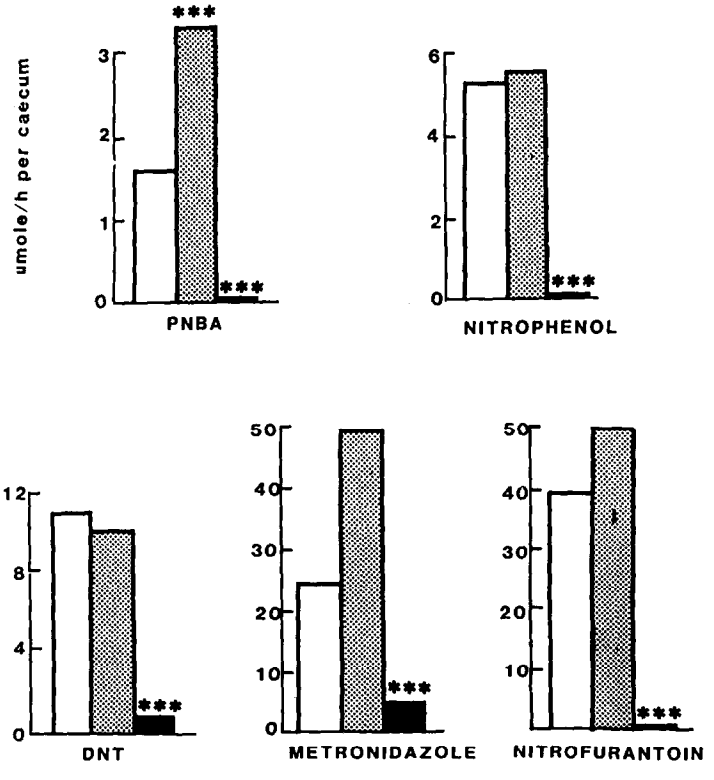

Fig. 5. Effect of dietary pectin and carrageenan on reduction of nitro compounds by rat caecal contents. For experimental details see Fig. 4. Key: PNBA, *p*-nitrobenzoic acid; DNT, 2,4-dinitrotoluene. Control fibre-free diet (□); 5% pectin diet (▨); 5% carageenan diet (■).

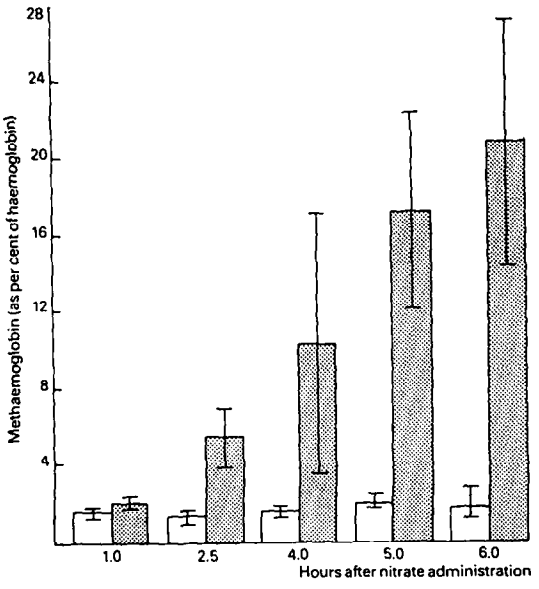

Fig. 6. Effect of dietary pectin on the development of methaemoglobinaemia in rats given sodium nitrate. Male Wistar rats were fed a purified fibre-free diet (□) or that diet supplemented with 5% pectin (▨) for 33 days. At zero time the animals were given a single oral dose of $NaNO_3$ (1.75 g/kg body weight). Methaemoglobin levels were determined on sequential samples of blood taken from the jugular vein. Values shown are the mean and S.E.M. for 6 control and 4 pectin-fed rats.

therefore, for improvements in the methods used for studying bacterial metabolism of foreign compounds, particularly to provide data of direct relevance to man. Several alternative approaches which attempt to model the human gut ecosystem *in vivo* or *in vitro* are currently under investigation at BIBRA. For example, it may be possible to increase the degree of similarity in gut flora metabolism between laboratory animals and man by modifying the animal diet. Rats, mice and hamsters fed a purified, fibre-free diet exhibit a greater similarity to man in terms of their caecal nitrate and nitro reductase activities than animals given stock laboratory diets (Table 3; [34]) although this may not be the case for other enzyme activities. We are also studying an animal model for human gut flora studies which utilizes germfree rats contaminated with human faecal organisms. This system allows *in vivo* metabolic and toxicological studies to be performed with animals whose caecal microflora possesses many microbial and enzymic properties characteristic of the human gut flora. Finally, by using a continuous flow culture system it is possible to model *in vitro*, the complex, mixed bacterial population of the mammalian gut and perform detailed studies of foreign compound metabolism over extended periods [35–37].

Table 3. Comparison of microbial enzyme activities in laboratory animals fed a purified diet with those of man

Species	Enzyme activities	
	Nitroreductase	Nitrate reductase
Man (N = 6)	0.79 ± 0.15	5.84 ± 1.74
Rat (N = 12)	1.39 ± 0.07	8.00 ± 1.00
Mouse (N = 4)	1.35 ± 0.08	4.90 ± 0.60
Hamster (N = 6)	0.83 ± 0.08	3.20 ± 0.90

Animals were fed a purified, fibre free diet.

Results shown are means ± S.E. of enzyme activities expressed as μmole/hr/g gut contents (caecal contents or human faeces).

From Rowland *et al.* 1983 and Rowland *et al.* unpublished observations (1985).

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