INTESTINAL BACTERIA AND THE HYDROLYSIS OF GLYCOSIDIC BONDS

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WITH the development of effective anaerobic techniques, the composition of the human intestinal bacterial flora is now more clearly understood. Although the detailed composition is dependent on the nature of the diet (see, for example, Hill *et al.*, 1971), in all cases studied the predominant faecal bacteria are those of the non-sporing strictly anaerobic groups. The metabolic significance of the gut flora is only slowly emerging, but a number of review articles (e.g., Smith, 1966; Scheline, 1968) attribute to it a wide range of reactions of toxicological interest.

The involvement of intestinal bacteria in such reactions is usually inferred from indirect evidence—for example, the effect of antibiotic treatment on the metabolism of the drug, or the comparison of germ-free and conventional animals—and there are few direct studies using pure strains of bacteria. In particular, the metabolic activities of the non-sporing strictly anaerobic bacteria are virtually unknown.

Many of the enzymes concerned in the metabolism of food additives and drugs are already well known to bacteriologists. For example, the β -glucosidase responsible for the hydrolysis of aesculin and salicin is also responsible for releasing the active agents from some cardiac glycosides (Lauterbach and Repke, 1960), from cascara sagrada (Fingl, 1965) and from cycasin (Spatz, McDaniel and Laqueur, 1966).

In the process of detoxification, many compounds are conjugated by the liver to form, for example, glucuronides, and then excreted in the bile into the intestine (Smith, 1966). The glucuronides may then be hydrolysed either by enzymes of the gut mucosa or by enzymes of any bacteria present in the gut, releasing the aglycone, which may be reabsorbed and may be subsequently re-excreted by the liver into the gut.

Escherichia coli is well known to produce a β -glucuronidase (Buehler, Katzman and Doisy, 1951), but *E. coli* normally constitutes only a small proportion of the gut flora and there are no quantitative studies on the relative contributions to the glucuronidase activity of the gut contents by various bacteria. Similarly the contributions of the different bacterial species to the total activity of other glycosidases in the gut have not been assessed.

We have therefore examined the gut flora of four laboratory animals commonly used in toxicological studies and estimated the activity of the glycosidases produced by the principal groups of intestinal bacteria.

MATERIALS AND METHODS

Characterisation of the gut flora. All the animals examined were fed *ad libitum*—rats and mice on diet 41B (Oxoid), rabbits and guinea-pigs on diet SG1. The rats, mice and guinea-pigs were killed with chloroform and the rabbits by intravenous injection of air. Immediately

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after death the abdomen was opened and the gastro-intestinal tract was ligatured at the oesophagus, at the duodenum at a point close to the stomach, at various points along the small intestine, at the ileo-caecal junction and at the rectum. After removal of the alimentary tract, 0.5-g amounts of the contents of the stomach and caecum were removed with sterile pasteur pipettes into 4.5-ml volumes of glycerol broth; the latter was 1 per cent. Lab Lemco (Oxoid) broth with 10 per cent. glycerol. The same amount of material was also removed from the upper small intestine, lower small intestine and rectum by gentle pressure after cutting the gut between the ligatures. All the specimens were stored in dry ice until cultivation (Drasar and Crowther, 1970). The bacteria and yeasts present were counted by the method described by Drasar (1967).

Reagents. All reagents were obtained from Koch-Light Ltd. Acetyl digoxin was prepared from lanatoside C by the action of β -glucosidase (Koch-Light Ltd).

TABLE I
The growth conditions for bacteria used in enzyme assays

Group of bacteria tested	Culture medium	Incubation conditions	
		Time (hr)	Atmosphere
Enterobacteria } Enterococci }	Nutrient broth no. 2 (Oxoid) +0.05 per cent. lactose	18	Air
Lactobacilli	PTYE medium*+0.05 per cent. lactose	48	80 per cent. N ₂ +20 per cent. CO ₂
Clostridia } Bacteroides } Bifidobacteria }	PTYE medium*+0.05 per cent. lactose	48	90 per cent. H ₂ +10 per cent. CO ₂

* PTYE medium = 10 g tryptone, 10 g soya peptone+10 g yeast extract per litre distilled water.

Enzyme assays. Fifty strains of each of the test bacterial species were grown in 20-ml amounts of medium under the conditions described in table I. Lactose broth was used for all the assays as the β -galactosidase of some organisms (e.g., *E. coli*) is inducible and is not produced if lactose or a similar inducer is absent or if glucose is present. The addition of sucrose did not noticeably stimulate β -glucosidase activity, nor was this enzyme repressed in the presence of lactose.

The bacteria were harvested by centrifugation and 3 ml of a washed-cell suspension in 0.025M phosphate buffer (pH 7.4) was used for each assay. The total number of organisms per ml of suspension was determined on the basis of the optical density at 600 nm and with reference to a standard plot of readings made for each of the test species of bacteria; the standard readings were made by direct enumeration of suspensions in a Helber counting chamber. 1 ml of the substrate solution at a concentration of 0.2 mg per ml was incubated with the bacterial suspension (3 ml) at 37°C for 3 hr, or for 24 hr when necessary (see text). The hydrolysis of *p*-nitrophenyl glycosides was assayed by the method of Kuby and Lardy (1953) and that of phenolphthalein glucuronide by the method of Fishman, Springer and Brunetti (1948).

The hydrolysis of aesculin (4,5-dihydroxycoumarin β -D-glucoside) was measured after incubation of 1 ml of bacterial suspension containing 10⁹ bacteria with 1 ml of a 1 per cent. solution of aesculin in phosphate buffer (pH 7.4, 0.025M) at 37°C for 1 hr; 2 ml of 0.5 per cent. ferric chloride solution was added and the optical density at 410 nm was recorded.

The cardiac glycoside, lanatoside C, was used as a further substrate for bacterial β -glucosidase. Bacterial suspensions were incubated with lanatoside C 0.5 mg per ml at 37°C

and pH 7.4 for 16 hr. The products were separated from the substrate by thin-layer chromatography on plates of silica gel G (Merck) subsequently developed with a mixture of hexane,

TABLE II

The composition of the intestinal flora of four laboratory animal species and man

Site and test animal species*	Mean log ₁₀ viable count† of the stated organisms							
	Entero-bacteria	Entero-cocci	Lacto-bacilli	Veil-lonellae	Yeasts	Clostridia	Bacteroides	Bifido-bacteria
Stomach								
Rabbit (3)	N‡	N	N	N	N	N	5.7	5.3
Guinea-pig (9)	N	N	3.9	N	N	3.3	N	5.9
Rat (8)	4.1	3.9	6.6	5.7	4.1	3.3	7.0	7.6
Mouse (3)	3.1	2.9	8.3	7.8	6.3	3.6	8.1	8.3
Man (30)	N	N	N	N	N	N	N	N
Upper small intestine								
Rabbit (3)	N	N	N	N	N	N	4.5	4.4
Guinea-pig (9)	N	N	3.5	N	N	3.2	N	5.1
Rat (14)	4.5	4.7	6.7	4.5	4.0	3.0	6.3	6.7
Mouse (3)	4.2	4.5	7.7	N	6.8	2.3	6.6	7.0
Man (30)	N	N	1.0	N	1.0	N	2.5	2.0
Lower small intestine								
Rabbit (3)	N	N	N	N	N	4.7	5.4	6.1
Guinea-pig (9)	N	N	4.0	N	N	3.4	6.3	6.0
Rat (8)	4.1	5.0	6.8	5.1	3.9	3.3	6.9	7.6
Mouse (3)	3.6	4.8	7.5	5.3	5.4	3.3	7.7	7.9
Man (5)	3.3	2.3	N	N	2.3	N	3.5	4.0
Large intestine								
Rabbit (3)	3.4	N	N	N	N	3.8	8.0	4.5
Guinea-pig (9)	N	3.2	4.3	4.0	N	3.1	7.1	8.4
Rat (8)	5.5	6.0	7.6	5.7	5.9	3.1	8.0	8.2
Mouse (3)	5.7	5.2	7.4	6.3	6.8	3.3	8.3	8.2
Man§ (11)	7.0	7.0	6.5	3.0	N	N	8.0	7.0
Rectum (and faeces)								
Rabbit (3)	2.3	2.3	4.0	N	N	3.8	7.8	7.7
Guinea-pig (9)	2.9	3.1	3.9	4.0	4.0	3.5	7.8	8.4
Rat (8)	5.9	6.6	7.8	5.6	5.8	3.0	8.2	8.6
Mouse (3)	5.0	4.8	8.1	6.6	6.6	3.3	8.4	7.8
Man (30)	6.0	3.5	4.0	3.0	1.0	3.0	10.5	10.5

* The number tested is given in brackets.

† The figures are the mean log₁₀ viable count per g wet weight of test material.

‡ N = less than 10 organisms per g wet weight of test material (see *Methods*).

§ After Seeliger and Werner (1963).

|| From Drasar, Shiner and McLeod (1969)

ethyl acetate and ethanol (15:75:10 v/v) (Nover *et al.*, 1969). Enzyme activity was estimated semiquantitatively by locating the products as blue spots with a 10 per cent. solution of phosphomolybdic acid in ethanol.

RESULTS

Intestinal flora of the laboratory animals

The results of our studies on the composition of the normal bacterial flora of the intestine of the rat, mouse, guinea-pig and rabbit are summarised in table II. For comparison, the normal intestinal flora of healthy persons investigated by Drasar, Shiner and MacLeod (1969) is indicated.

Rats and mice are coprophagic and so ingest large numbers of bacteria. These animals have large numbers of bacteria along the whole length of the gastro-intestinal tract. In the stomach and small intestine the most numerous organisms are lactobacilli, *Bacteroides* species and bifidobacteria; these organisms outnumber the enterobacteria (mainly *Escherichia coli*) by a factor of more than 10^2 . In contrast, the stomach and small intestine of the rabbit and guinea-pig contain a very simple flora, similar to that of man, and the total number of bacteria is much lower than in the mouse and rat.

In the large intestine a more complex and profuse flora is present in all four animal species. The principal groups of bacteria present are the enterobacteria (mainly *E. coli*), enterococci, lactobacilli, *Bacteroides* species and bifidobacteria; in all the animals the strictly anaerobic bacteria (*bacteroides* and bifidobacteria) greatly outnumber the aerobes (enterobacteria and enterococci), with lactobacilli present in very large numbers in the rat and mouse, and in smaller numbers in the rabbit, guinea-pig and man.

Enzyme activity of gut bacteria

Pure cultures of 50 strains representing six groups of bacteria—*E. coli*, enterococci, lactobacilli, Nagler-positive clostridia, *Bacteroides* species and bifidobacteria—were assayed for the production of β -glucuronidase, α - and β -glucosidases and α - and β -galactosidases. The results of the assays involving nitrophenyl substrates are presented as a series of histograms (figure) and the mean values, expressed as μ moles nitrophenol released per hr per 10^8 bacteria, are given in table III. Enterococci produce large amounts of β -glucosidase and β -galactosidase, but little β -glucuronidase, whereas *E. coli* produces large amounts of β -glucuronidase and β -galactosidase, but little β -glucosidase. The non-sporing anaerobes produce intermediate amounts of all of these enzymes except β -glucuronidase.

β -glucuronidase was produced in greatest amount by *E. coli*; the Nagler-positive clostridia and *Bacteroides* species produced an intermediate amount of this enzyme, and the enterococci, lactobacilli and bifidobacteria produced very little.

Similar results were obtained with phenolphthalein substrates and with aesculin as a substrate for β -glucosidase. The cardiac glycoside, lanatoside C, was hydrolysed to acetyl digoxin by more of the enterococci than of the other species. Some enterococcal strains also degraded this secondary glycoside, removing the O-acetyl group and yielding digoxin.

DISCUSSION

The extent to which compounds, whether ingested or excreted in the bile, are metabolised by gut bacteria depends on at least three factors—(i) the

FIGURE.—A comparison of the various enzyme activities of 50 test strains representing six different groups of intestinal bacteria.

region of the gut from which the compound is absorbed; (ii) the distribution of bacteria along the gut; and (iii) the occurrence of the necessary enzyme or

enzyme system in the bacteria. The principal site of absorption of hydrolysed compounds is the small intestine, and this region of the gut is much less heavily colonised in man and the rabbit than in the other animals tested, so that compounds are less likely to be degraded by bacteria in the human or rabbit gut than in the gut of the rat or mouse.

In the rabbit, the pH value of the stomach contents is normally sufficiently low to be bacteriostatic and sometimes low enough to be bactericidal (Smith, 1965). In guinea-pigs that have been actively feeding, the pH of the stomach contents (mean 3.5) is high enough to allow the growth of lactobacilli and

TABLE III
Mean activity of the glycosidases of the principal species of gut bacteria

Enzyme assayed	Mean value (μ moles substrate degraded per hr per 10^8 cells) for 50 test strains in each of the stated groups					
	Entero-bacteria	Enterococci	Lactobacilli	Clostridia	Bacteroides	Bifido-bacteria
β -Galactosidase	42.4 $\pm$ 3.2*	53.8 $\pm$ 6.0	90.6 $\pm$ 10.7	13.7 $\pm$ 2.7	50.7 $\pm$ 4.9	39.1 $\pm$ 4.7
β -Glucosidase	5.8 $\pm$ 2.5	192.7 $\pm$ 19.5	26.0 $\pm$ 7.4	22.1 $\pm$ 5.0	35.1 $\pm$ 4.8	29.3 $\pm$ 6.0
β -Glucuronidase	24.7 $\pm$ 2.1	2.9 $\pm$ 0.6	1.6 $\pm$ 0.2	11.3 $\pm$ 2.3	6.0 $\pm$ 3.5	1.9 $\pm$ 0.8
α -Galactosidase	12.7 $\pm$ 2.1	20.8 $\pm$ 4.3	97.7 $\pm$ 12.1	53.1 $\pm$ 8.1	24.0 $\pm$ 4.8	28.2 $\pm$ 3.8
α -Glucosidase	5.9 $\pm$ 0.5	14.0 $\pm$ 1.1	26.6 $\pm$ 3.5	30.1 $\pm$ 6.4	9.8 $\pm$ 2.0	20.7 $\pm$ 3.0

* The standard error of the mean is given in each case.

bifidobacteria (the organisms least affected by acid conditions), but rats and mice secrete much less gastric acid, so that the ingested flora is not destroyed and there is considerable evidence for multiplication of bacteria in the mouse stomach (Dubos *et al.*, 1965). In the rat and mouse there is a constant flow of bacteria into the intestine, which may partly account for its being more heavily populated than the intestine of the rabbit, guinea-pig or man.

Glucuronide formation is a major detoxification mechanism in animals, and it is probable that glucuronides excreted in the bile are deconjugated by bacterial β -glucuronidase. For example, there is evidence that stilboestrol glucuronide is hydrolysed mainly by the action of bacterial enzymes in the gut (Dr Millburn, personal communication).

An approximate estimate of the total β -glucuronidase activity derived from each group of bacteria can be made by multiplying the mean activity observed per cell by the number of the bacteria present, assuming that the bacterial enzymes of the different species have the same potential activity in the intestine as that observed *in vitro*. The results of such a calculation (table IV) suggest that, in the rat, glucuronides are hydrolysed principally by the non-sporing

anaerobes and the lactobacilli, although in the proximal small intestine *Escherichia coli* may make some contribution. A similar calculation reveals

TABLE IV
Estimated bacterial β -glucuronidase activity in the small intestine of the rat

Test group of bacteria	Mean activity per 10^8 cells*	Estimates for proximal small intestine		Estimates for distal small intestine	
		Number of bacteria	Total activity†	Number of bacteria	Total activity†
Enterobacteria	24.7	3.2×10^4	7.9	1.3×10^4	3.2
Enterococci	2.9	5.0×10^4	1.5	1.0×10^5	2.9
Lactobacilli	1.6	5.0×10^6	80.0	6.3×10^6	101.0
Clostridia	11.3	1.0×10^3	0.1	2.0×10^3	0.2
Bacteroides	6.0	2.0×10^6	120.0	7.9×10^6	474.0
Bifidobacteria	1.9	5.0×10^6	95.0	4.0×10^6	760.0
Cumulative total estimate of β -glucuronidase activity	...	...	304	...	1341

* See table III.

† μ moles substrate degraded per hr per g intestinal contents.

that although the enterococci are the organisms that produce the highest level of β -glucosidase per cell, the organisms most likely to be responsible for hydrolysis in the small intestine of the rat are again the strict anaerobes.

TABLE V
Estimated bacterial β -glucuronidase activity in the small intestine of man and four laboratory animal species

Animal species	Estimated β -glucuronidase activity*	
	in proximal small intestine	in distal small intestine
Man	0.02	0.9
Rabbit	2.4	45.4
Guinea-pig	2.7	139
Rat	304	1341
Mouse	1200	5015

* μ moles substrate degraded per hr per g intestinal contents.

The level of β -glucuronidase activity in the small intestine of man, rabbit and guinea-pig is very low compared with that observed in the rat (table V). This is in agreement with the results of Marsh, Alexander and Levvy (1952),

who showed that the β -glucuronidase activity of ileal contents of the rat was 15 times greater than that of the ileal contents of the rabbit. This suggests that hydrolysis, and therefore hepatic recycling, of glucuronides occurs much more extensively in the small intestine of the rat and mouse (the animals most commonly used in toxicological studies) than in man.

SUMMARY

As part of a study of the metabolic activities of intestinal bacteria, the activity of the principal glycosidases produced by the major groups of intestinal bacteria has been estimated. The results are discussed in terms of the toxicological and pharmacological effects of various ingested glycosides and of the enterohepatic circulation of compounds by four laboratory animal species commonly used in toxicological studies.

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