Metabolic Activity and Enzyme Induction in Rat Fecal Microflora Maintained in Continuous Culture

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The enzyme activity of the rat hindgut microflora maintained in an anaerobic two-stage continuous culture was compared with that of rat cecal contents. A qualitative comparison (API ZYM) showed a high degree of similarity between the two populations. Quantitative determinations showed that azoreductase, β-glucosidase, nitrate reductase, and nitroreductase activities were comparable, and that β-glucuronidase activity was very low in the culture. β-Glucuronidase, β-glucosidase, and nitrate reductase activities were induced within the culture by their respective substrates. Bile acids influenced microbial activity in vitro, with cholic acid inducing β-glucosidase, azoreductase, and β-glucuronidase activities and decreasing nitrate reductase activity. Chenodexycholic acid increased β-glucosidase and β-glucuronidase activities and decreased azoreductase, nitrate reductase, and nitroreductase activities in vitro. These studies demonstrate that the rat hindgut microflora may be successfully cultured in vitro and suggest control mechanisms that regulate the metabolic activity of these organisms in vivo.

The growth characteristics of rat hindgut microflora can be simulated in vitro by an anaerobic two-stage continuous culture system with cell recycling (17). The mixed bacterial population that develops under these conditions closely resembles that of the original fecal inoculum in terms of species diversity and the relative proportions of the major groups of organisms present in the culture (17). This system permits the long-term investigation in vitro of the ecology of the hindgut microflora over prolonged periods under defined conditions similar to those found in vivo.

An important application for the in vitro culture is in the study of the metabolic activity of the flora, which may be investigated without the confounding effects of the competing enzymes of the host animal. In addition to its metabolic activities related to the maintenance and growth of the cell (16), the mammalian gut flora catalyzes many reactions (often hydrolytic or reductive) involving a wide range of anutrient or xenobiotic compounds (4, 15). These reactions may be of importance to the host animal and can result in the activation or detoxication of foreign compounds (13, 19).

One of the attributes of the intestinal flora is its ability to adapt to metabolic pressure in the form of novel substrates. This adaptive ability has been exemplified by the induction of sulfamatase activity in the gut bacteria with the conversion of cyclamate to cyclohexylamine (1).

Adaption has also been reported with the pyrimidine precursor orotic acid (20). However, few examples of metabolic adaption with the gut bacterial flora have been reported.

In this communication we report the activities of a variety of enzymes present in the hindgut flora maintained in continuous culture. We also describe the effects of bile acids on bacterial metabolism.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (approximately 150 g) were purchased from Olac (1976) Ltd. (Bicester, Oxon, United Kingdom), and feces were used as the source of inocula for the continuous culture. The animals were allowed free access to tap water and laboratory animal diet no. 1 (Spratts Patent Ltd., Barking, Essex).

Culture system and bacteriological methods. A two-stage continuous culture system with cell recycling was inoculated and maintained as described by Veilleux and Rowland (17). The culture was allowed to stabilize for 14 days before any investigations into bacterial metabolic activities. All other bacteriological methods were as described previously (17). The production of short-chain fatty acids in stages 1 and 2 of the continuous culture was determined (7) from the time of inoculation until day 24.

Determination of microbial metabolic activities. For semiquantitative determination of a range of microbial enzymes present in the continuous culture and the native cecal microflora, we utilized the API ZYM system (API Products Ltd. Montalieu Vercieu, France). The system identified 19 common bacterial

reactions (see Fig. 2) by the visual estimation of chromophoric products. A sample of medium from stage 2 of the continuous culture was diluted 1:1 with normal saline and used as the source of enzyme. Preparations of the rat cecal microflora were suspended in this same medium as described by Wise et al. (21). Samples (65 μl) of each preparation were added to the individual cuvettes and incubated at 37°C for 4 h. The intensity of color for each cuvette was estimated in natural light by comparison with a chart of standards.

Quantitative determinations of azoreductase (amaranth), nitroreductase (nitrobenzoic acid), and nitrate reductase (sodium nitrate) activities (21) and β-glucuronidase (p-nitrophenol-β-D-glucuronide) and β-glucosidase (p-nitrophenyl-β-D-glucoside) activities (14) were by published methods. The rate of metabolism of these compounds was also determined in vitro after the introduction of a single sample of substrate into stage 2 of the culture system (final concentration, 0.5 mM).

Effects of bile acids on microbial metabolic activities. Cholic acid and chenodeoxycholic acid (sodium salt, Sigma Chemical Co., Poole, Dorset, United Kingdom) were dissolved in deionized water and continuously added to stage 2 of the culture system (0.35 ml/h) with a syringe pump (Sage Instruments, Cambridge, Mass.) to a steady-state concentration of 10 μg/ml. Bacterial azoreductase, β-glucuronidase, β-glucosidase, nitrate reductase, and nitroreductase activities were determined as described above.

RESULTS

Production of short-chain fatty acids. Ethanoic, propanoic, butanoic, and pentanoic acids were detected within stage 1 (pH 5.2) of the culture system 24 h after inoculation, although 2-methylpropanoic and 3-methybutanoic acids were not detected until 7 days (Fig. 1). The concentration of ethanoic and propanoic acid stabilized approximately 10 days after inoculation, whereas the production of the remaining five components continued to increase over the 24 days of the study. In stage 2 (pH 7.4) of the system, ethanoic and propanoic acids were detected 24 h after inoculation, but the remaining straight-chain and branched-chain acids were not found until day 2 (Fig. 1). The concentration of these products attained a steady-state value approximately 7 to 10 days after inoculation, with the exception of hexanoic acid, which continued to increase over the period of the study.

Enzyme profile of rat cecal microflora. The hydrolytic enzymes activities in stage 2 of the continuous culture were assessed with the API ZYM system and were broadly similar to those present in rat feces (Fig. 2). Preliminary studies showed no interference from components in the culture medium with color development in the assays, and the generation of reaction products was abolished after heating the bacterial preparation in a boiling water bath for 10 min. Lipase,
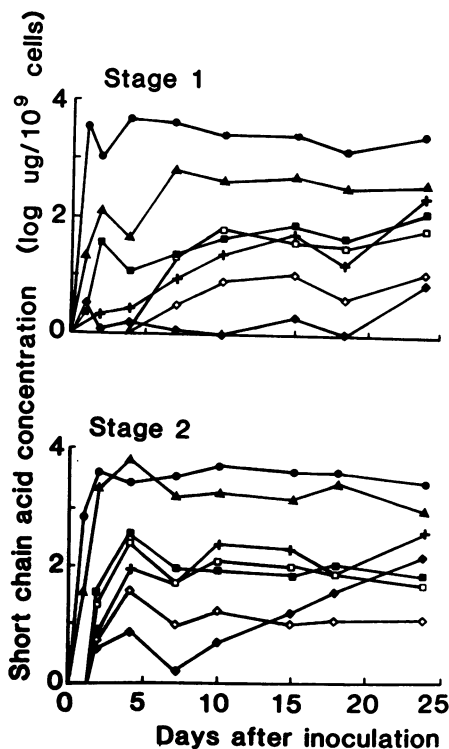

FIG. 1. Production of short-chain fatty acids by the rat hindgut microflora maintained in continuous culture. Symbols: ●, ethanoic acid; ▲, propanoic acid; ■, butanoic acid; +, pentanoic acid; ◆, hexanoic acid; □, 2-methylpropanoic acid; ◇, 3-methylbutanoic acid.

N-acetyl-β-glucosamidase, and α-fucosidase activities were not detected in the fecal microflora in vitro, but were present in cecal contents, whereas β-glucuronidase was relatively less active in the continuous culture. The enzyme activities present at high activity in the cecal preparation, such as alkaline phosphatase, leucine arylamidase, α-galactosidase, β-galactosidase, and β-glucosidase, were also predominant in the continuous culture. α-Mannosidase activity was not detected in cecal contents, but was present at low activity in the culture.

Quantitative comparison of bacterial enzyme activities. The metabolic activity of the microbial population from stage 2 of the continuous culture was similar to that of the cecal microflora (Table 1) when enzyme activities were standardized on the basis of a constant number of bacteria (10^{11}). The culture contained 2.09×10^9 bacteria per ml, whereas the population for rat cecal contents was 5.75×10^{10} organisms per g. Nitrate reductase and nitroreductase activities were greatest in the continuous culture, and β-glucosidase was most active in cecal contents,
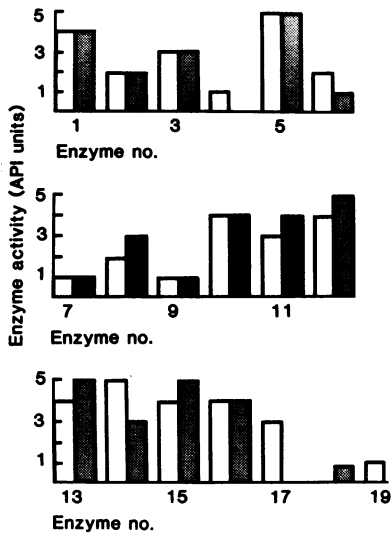
FIG. 2. Comparison of bacterial enzyme activities. A qualitative comparison of bacterial enzyme activities was made with the API ZYM system as described in the text. Symbols: □, cecal contents ($n = 3$); ▨, continuous culture ($n = 3$). Enzymes: 1, alkaline phosphatase; 2, esterase (C4); 3, esterase lipase (C8); 4, lipase (C14); 5, leucine arylamidase; 6, valine arylamidase; 7, cysteine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase; 11, phosphoamidase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, N-acetyl-β-glucosaminidase; 18, α-mannosidase; 19, α-fucosidase.

yet all were within the same order of magnitude. The major difference in enzyme content of the two preparations was β-glucuronidase activity, which was 30-fold more active in fresh cecal contents than the continuous culture.

Induction of enzyme activities within the rat hindgut microflora. β-Glucuronidase, β-glucosidase, and nitrate reductase activities were induced within the rat fecal microflora after the addition of the appropriate substrate to the continuous culture (Fig. 3). β-Glucosidase and ni-

TABLE 1. Comparison of microbial metabolic activities in cecal contents and continuous culture

Enzyme	Activity[a]	
	Cecal contents ($n = 3$)	Continuous culture ($n = 4$)
Azoreductase	3.5 ± 0.3	2.1 ± 0.5
β-Glucosidase	5.9 ± 0.3	2.3 ± 0.1
β-Glucuronidase	27.3 ± 2.1	1.1 ± 0.1
Nitrate reductase	6.8 ± 0.7	10.2 ± 0.5
Nitroreductase	2.1 ± 0.2	4.2 ± 0.5

[a] Micromoles of substrate transformed per hour per 10^{11} bacteria. Values are given as means ± standard deviations of group results.
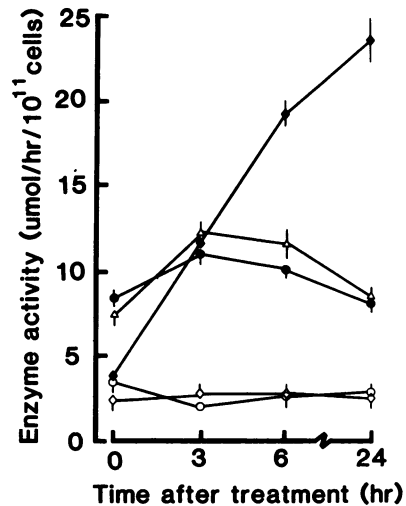

FIG. 3. Substrate induction of enzyme activity in rat fecal microflora in continuous culture. The appropriate substrate (0.5 mM, final concentration) was added to the culture at zero time, and the metabolic activity of the fecal microflora was determined 0, 3, 6, and 24 h after treatment. Values are given as means ± standard deviations ($n = 4$). Symbols: ○, azoreductase; △, β-glucosidase; ◆, β-glucuronidase; ●, nitrate reductase; □, nitroreductase.

trate reductase activities reached a maximum (increased by 20 and 50%, respectively) approximately 3 h after treatment, yet β-glucuronidase activity continued to increase and was elevated eightfold after 24 h. Azoreductase and nitroreductase substrates failed to induce their own metabolism within the culture.

The addition of cholic acid to the continuous culture gave an initial decrease in activity of several of the enzymes measured, yet β-glucosidase and azoreductase activities increased over the course of the study (Fig. 4). β-Glucuronidase activity was not detected in this culture at zero time, but was found at significant levels after 7 days of exposure to cholate. Nitrate reductase activity was decreased by cholate treatment, whereas nitroreductase activity was relatively unchanged. Before the introduction of cholate, azoreductase and nitroreductase activities varied by 1 to 5% over a 3-day period, whereas β-glucosidase and nitrate reductase activities showed larger daily variations (10 and 15%, respectively). β-Glucuronidase activity was not detected or was extremely low over this period. Chenodeoxycholic acid gave less consistent changes in microbial enzyme activities, although β-glucuronidase and β-glucosidase activities were increased and azoreductase and nitroreductase activities decreased 7 days after the start of treatment (Fig. 4).
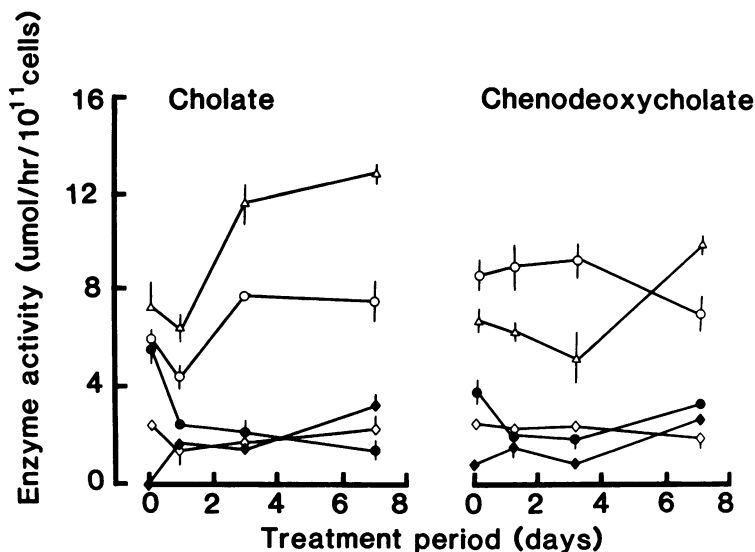
FIG. 4. Effect of bile acids on enzyme activity of rat fecal microflora in continuous culture. Cholic acid or chenodeoxycholic acid (10 μg/ml, final concentration) was added continuously to the culture system, and the metabolic activity of the fecal microflora was determined after 0, 1, 3, and 7 days. Values are given as means ± standard deviations (n = 3). Symbols: ○, azoreductase; △, β-glucosidase; ◆, β-glucuronidase; ●, nitrate reductase; □, nitroreductase.

DISCUSSION

The rat hindgut microflora may be maintained in vitro for extended periods in a two-stage continuous culture, with the bacterial population stabilizing in terms of numbers of organisms and relative species diversity 10 to 14 days after inoculation (17). An additional indicator of stability within this bacterial community is the production of short-chain fatty acids. These materials are the end products of a number of fermentative microbial pathways (16), and their presence in the culture medium is related to bacterial growth (7). In the present study production of the majority of the short-chain acids in stage 2 of the culture stabilized approximately 10 days after inoculation, indicating that bacterial metabolism and bacterial numbers are closely linked.

Fecal bacterial enzyme activities within the continuous culture showed a range of hydrolytic and reductive functions similar to that found in cecal contents, suggesting an overall concordance in microbial metabolism within the two bacterial populations. Further, the rate of metabolism of a number of "model" xenobiotic substrates was comparable for the in vivo- and in vitro-derived microflora, although β-glucuronidase activity was lower in the culture system. This enzyme, however, was induced within the culture after the addition of a β-glucuronide, and the resulting activity was similar to that of cecal contents.

The substrate-induced increases in activities of β-glucuronidase and β-glucosidase, together with that of nitrate reductase, provide examples of the ability of the intestinal flora to adapt to changes in environmental and dietary factors. Microbial metabolism in the gut may be regulated by exogenous factors like nitrate and glucosides, which are present in many foods, especially those of plant origin, and by endogenous factors such as glucuronides (the products of mammalian hepatic conjugation reactions [2]). These results also illustrate the advantages of the continuous culture system in studies of bacterial metabolic adaptation, especially toward endogenously produced substances such as glucuronides. Attempts to study the induction of β-glucuronidase activity in vivo or in batch culture by using gut contents freshly isolated from the animal would be complicated by the presence of the endogenously synthesized substrates.

Bacterial enzyme activity in the culture system was also influenced by bile acids, which are mammalian products also present in the gastrointestinal tract. Cholic acid, present as its taurine conjugate (8), is the major steroid present in rat bile, comprising approximately 40% of the total excreted (11). Hepatic excretion of bile acids may be increased by a high-fat diet and decreased by dietary fiber (10), suggesting a potential mechanism for the environmental control of bacterial metabolism. In addition the

activity of certain of the enzymes was increased by bile acid treatment in vitro (β-glucuronidase, azoreductase), and these enzymes have been implicated in the development of cancer in the lower gastrointestinal tract (3, 18).

Methods currently used for studying the metabolic activity of the gut flora have various disadvantages. For example, the use of germ-free or antibiotic-treated animals to assess the relative contribution of mammalian and microbial enzymes in the disposition of compound in vivo (5, 9, 12) is complicated by the physiological and metabolic abnormalities of these animals in comparison to their conventional counterparts (4, 6). Maintenance of the intestinal flora in batch culture in vitro for relatively short periods results in physicochemical changes in the medium and distorts the relative proportions of the groups of organisms (4, 16; I. R. Rowland and R. Walker, *In* D. M. Conning and A. Lansdown, ed., *Toxic Hazards in Food*, in press), leading to metabolic activity that is not representative of that occurring in the gut in vivo.

The continuous culture system described in this publication enables a stable gut population to be maintained in vitro, dissociated from the influences of the host animal. The system will therefore facilitate investigations of intestinal bacterial enzyme activity over prolonged periods and facilitate the study of factors controlling bacterial metabolism in the gut.

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