Influence of pH, Nutrient Availability, and Growth Rate on Amine Production by Bacteroides fragilis and Clostridium perfringens

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Dimethylamine, methylamine, propylamine, and pyrrolidine were the major amines formed by Bacteroides fragilis NCDO 2217 during the active phase of growth in batch culture. Production of these metabolites was strongly pH dependent and was optimal under acidic conditions (pH 6.0). Low pH also favored the formation of pyrrolidine, cadaverine, and dimethylamine by Clostridium perfringens CS23, but the reverse was the case with putrescine, butyamine, and propylamine, where production was maximal at neutral pH. B. fragilis was grown in continuous culture under either starch or casein limitation. Amine formation was influenced by carbohydrate availability and was greatest when the bacteria were grown at high growth rates (dilution rate, 0.20/h) under starch limitation, where they constituted about 18% of the total fermentation products measured. Amine production was optimal and increased concomitantly with growth rate when C. perfringens was grown in glucose-limited continuous culture. Under conditions of high growth rate and glucose limitation, amines accounted for approximately 27% of the fermentation products measured. When glucose in the feed medium was increased from 5 to 15 g/liter, amine production was repressed, and under these nutritional conditions the growth rate had little effect on the process.

Nitrogen enters the human large intestine mainly in the form of proteins and peptides (6). After hydrolysis of the macromolecules by colonic proteases and peptidases, either intestinal bacteria can deaminate the amino acids to form a variety of products, including ammonia, carboxylic acids, indoles and phenols (25), or the amino acids may be decarboxylated to produce amines and CO2 (13, 14, 23). Amines can also be formed by N-dealkylation reactions (16), degradation of polyamines (32), or transamination of aldehydes (10).

Amines produced as a result of the activities of bacteria in the large gut may have physiological effects on the host. For example, putrescine and cadaverine are weak pressor substances (10), whereas it has been reported that amine concentrations are significantly higher in feces from infants with gastroenteritis compared with concentrations in feces from their healthy counterparts (25). More importantly, however, amines may play a role in colon cancer through the formation of N-nitrosamines in the gut (30, 33). These substances are formed chemically by the condensation of a secondary amine with nitrite under acidic conditions (29), or the reactions can be catalyzed by bacterial enzymes at neutral pH (5, 30, 31).

Many colonic bacteria (bacteroides, clostridia, bifidobacteria, enterobacteria, streptococci) are known to produce amines (3, 4, 10, 23). The enterobacteria (13, 23) and clostridia (16), in particular, have been shown to form these metabolites in large quantities. However, comparatively little is known about amine production in the colon, particularly with respect to the physiological and nutritional factors that influence the process.

In this paper, we report the results of studies on two species of intestinal anaerobes, Bacteroides fragilis and Clostridium perfringens, in which the effects of pH, nutrient availability, and growth rate on amine formation were investigated. B. fragilis was chosen because of the numerical predominance of bacteroides in the colon, whereas C. perfringens was selected as a representative high-amine-producing bacterium.

MATERIALS AND METHODS

Bacteria. B. fragilis NCDO 2217 was obtained from the National Institute for Research in Dairying, Shinfield, Reading, United Kingdom. C. perfringens CS23 was isolated from human feces by using direct plating procedures (20) and identified according to Gram stain, cell morphology, and fermentation products formed after growth in peptone-yeast-glucose medium (15). Characterization to species level was established on the basis of biochemical reactions on API 20A test strips (Analytab Products) and lecithinase production (18).

Batch cultures. Bacteria were grown in three identical glass fermentation vessels (0.3-liters working volume). C. perfringens was grown in a medium containing 1 ml of trace elements solution (11) plus the following (grams per liter): K2HPO4, 5.2; KH2PO4, 5.6; NaCl, 4.5; MgSO4·7H2O, 0.4; CaCl2·2H2O, 0.1; cysteine, 2.5; glucose, 5.0; yeast extract, 2.5; peptone water, 10.0. The medium was autoclaved at 121°C for 15 min and cooled under high-purity nitrogen gas. The bacteria were incubated at 37°C, and culture pH (7.0, 6.5, 6.0) was controlled as described previously (21). Anaerobic conditions were maintained during growth by sparging cultures with a gas mixture containing H2-CO2-N2 (10:10:80). B. fragilis was grown similarly, except that the growth medium was Wilkins-Chalgren broth. Samples were taken from cultures periodically for measurements of bacterial growth and fermentation products.

Continuous culture studies. The bacteria were grown in continuous culture in 0.5-liter (working volume) glass chemostats. Temperature (37°C), pH (6.8), and anaerobic conditions were maintained as described previously. B. fragilis was grown on a casein-limited basal mineral salts medium (1) containing casein (1.5 g/liter) and starch (10 g/liter) as carbon source.
g/liter). In the starch-limited medium, casein and starch concentrations were 1.5 and 1.0 g/liter, respectively. The medium used in continuous cultures of *C. perfringens* contained 1 ml of trace elements solution plus the following (grams per liter): K₂HPO₄, 5.2; KH₂PO₄, 5.6; NaCl, 4.5; MgSO₄ · 7H₂O, 0.4; CaCl₂ · 2H₂O, 0.1; cysteine, 2.5; yeast extract, 1.0; peptone water, 5.0. Glucose concentrations were 5.0 and 15.0 g/liter in the glucose-limited and peptide-limited media, respectively.

**Dry weight and chemical analysis.** Culture dry weights were determined as described by Keith and Herbert (17). Before chemical analysis, bacteria were removed from samples by centrifugation at 27,000 × g (8 min). Volatile fatty acids and other carboxylic acids were detected by gas chromatography by the method of Holdeman et al. (15). Samples for amines were stored frozen in 0.1 M HCl before analysis. The amines were measured with a Pye model 204 gas chromatograph fitted with a flame ionization detector connected to a Pye-CDP 1 computing integrator. The glass column (1.8 m by 2 mm [inner diameter]) contained GP Carbopack B-4% Carbowax 20 M-0.8% KOH. The respective flow rates of the nitrogen carrier gas, hydrogen, and air were 35, 30, and 370 ml/min. The injection port and detector were maintained at 150 and 200°C, respectively. The temperature of the column oven was controlled by a program as follows: 5 min at 80°C, followed by a temperature increase of 8°C/min up to 195°C. Samples (0.5 ml) to be measured were added to an equal volume of a solution containing NaOH (2%, wt/vol) and NH₄OH (2%, wt/vol). Portions (1 μl) were injected onto the column, and individual amines were identified and quantified by comparison of their retention times with those of authentic amine standards.

**Chemicals.** All chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, United Kingdom), except the formulated bacteriological media, which were purchased from Oxoid Ltd. (London, United Kingdom).

**RESULTS**

**Effect of pH on growth and amine production by *B. fragilis*.** Culture pH in the range of 6.0 to 7.0 had little effect on growth rates or cell yields in batch cultures of *B. fragilis* (Fig. 1a). However, amine formation was strongly stimulated during growth at low pH. Amine production by *B. fragilis* was closely associated with the growth cycle and occurred primarily in exponentially growing cultures. Methylvamine, dimethylamine, and propylamine were the predominant amines detected at all pH values after 24 h of incubation (Table 1).

**Influence of pH on growth and amine production by *C. perfringens*.** Growth rates of *C. perfringens* were almost identical over the pH range of 6.0 to 7.0, although final cell yields were slightly higher in cultures grown at pH 6.0 (Fig. 1b). Whereas the initial rates of amine formation were higher when the bacteria were grown under acidic conditions, total amine production after 24 h was greatest at pH 7.0. This was in large part accounted for by propylamine, which was the principal amine formed at all culture pHs tested. The production of this amine was markedly influenced by pH, and its percentage contribution to total amines increased concomitantly with pH, from 44% at pH 6.0 to 61% at pH 7.0 (Table 1). Whereas the formation of propylamine, putrescine, and butylamine was optimal at pH 7.0, pyrrolidine, dimethylamine, and cadaverine production occurred maximally at pH 6.0. Culture pH had little effect on methylamine formation (Table 1).

**Effect of growth rate and nutrient availability on amine production in continuous cultures of *B. fragilis*.** The bacteria were grown under carbohydrate (starch) and nitrogen (casein) limitation at various growth rates. Concentrations of acidic fermentation products were higher in nitrogen-limited cultures than in those grown under starch limitation (Table 2). The major amines produced by *B. fragilis* in continuous culture were dimethylamine, propylamine, and pyrrolidine. Amine production in the casein- and starch-limited chemostats was markedly influenced by growth rate, both with respect to total levels of amines formed and in their percentage contribution toward total fermentation products. Amine concentrations under starch-limited conditions at dilution rates (Ds) of 0.03/h and 0.20/h were 0.3 and 2.1 mmol/g (dry weight) of cells, constituting 3 and 18% of total fermentation products formed, respectively. The effect of growth rate on amine production was less marked in casein-limited chemostats, where total amines were 0.6 and 1.4 mmol/g (dry weight) of cells at Ds of 0.03/h and 0.02/h, respectively, accounting for about 5 and 8% of total fermentation products.

**TABLE 1. Effect of pH on the production of primary, secondary, and polyamines by *B. fragilis* and *C. perfringens*.**

<table>
<thead>
<tr>
<th>Species and pH of medium</th>
<th>Amines (mM)</th>
<th>Me</th>
<th>Di</th>
<th>Pr</th>
<th>Py</th>
<th>Bu</th>
<th>Pu</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. fragilis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0.57</td>
<td>0.68</td>
<td>0.15</td>
<td>0.08</td>
<td>T</td>
<td>T</td>
<td>ND</td>
<td>1.48</td>
</tr>
<tr>
<td>6.5</td>
<td>0.30</td>
<td>0.35</td>
<td>0.12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.77</td>
</tr>
<tr>
<td>7.0</td>
<td>0.20</td>
<td>0.28</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.55</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0.87</td>
<td>0.08</td>
<td>1.27</td>
<td>0.35</td>
<td>0.07</td>
<td>0.05</td>
<td>0.21</td>
<td>2.90</td>
</tr>
<tr>
<td>6.5</td>
<td>0.83</td>
<td>ND</td>
<td>1.58</td>
<td>0.17</td>
<td>0.07</td>
<td>0.15</td>
<td>0.15</td>
<td>2.95</td>
</tr>
<tr>
<td>7.0</td>
<td>0.97</td>
<td>ND</td>
<td>2.33</td>
<td>ND</td>
<td>0.25</td>
<td>0.18</td>
<td>0.10</td>
<td>3.83</td>
</tr>
</tbody>
</table>

* Bacteria were grown anaerobically in batch culture for 24 h at 37°C. Me, methylvamine; Di, dimethylamine; Pr, propylamine; Py, pyrrolidine; Pi, pyrrolidine; Bu, butylamine; Pu, putrescine. T, Trace (<0.1 mM); ND, not detected.
TABLE 2. Influence of carbohydrate availability and growth rate on the production of organic acids and amines by B. fragilis grown in continuous culture

<table>
<thead>
<tr>
<th>$D$ (per h)</th>
<th>Culture conditions</th>
<th>Organic acids</th>
<th>Amines</th>
<th>% Amines produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>P</td>
<td>IV</td>
</tr>
<tr>
<td>0.03</td>
<td>Starch limited</td>
<td>5.9</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Casein limited</td>
<td>8.0</td>
<td>3.8</td>
<td>T</td>
</tr>
<tr>
<td>0.10</td>
<td>Starch limited</td>
<td>6.4</td>
<td>2.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Casein limited</td>
<td>8.9</td>
<td>3.6</td>
<td>ND</td>
</tr>
<tr>
<td>0.20</td>
<td>Starch limited</td>
<td>6.2</td>
<td>1.4</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>Casein limited</td>
<td>9.5</td>
<td>2.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cultures were grown on a starch-limited medium containing 1.5 g of starch per liter and 1.5 g of casein per liter or on a casein-limited medium containing 10.0 g of starch per liter and 1.5 g of casein per liter.

** A, Acetate; P, propionate; IV, isovalerate; S, succinate; Me, methylvamine; Di, dimethylamine; Pr, propylamine; Py, pyrrolidine; Pi, piperidine; Mb, 2-methylbutylamine; Pu, putrescine; Ca, cadaverine. ND, Not detected; T, trace (<0.1 mmol/g [dry weight] of cells)

Influence of growth rate and nutrient availability on amine production in continuous cultures of C. perfringens.

The bacteria were grown in glucose-limited and peptide-limited chemostats at various growth rates. Total levels of fermentation acids, especially lactate, were greatest in peptide-limited cultures (Table 3). The acidic fermentation product balance was markedly influenced by dilution rate in both cultures as shown by the accumulation of lactate and the reduction in acetate formation at higher growth rates. More amines were produced by glucose-limited bacteria, particularly at a $D$ of 0.16/h (Table 3). Amine production was growth rate dependent in bacteria grown under glucose-limited conditions but not in bacteria grown in peptide-limited conditions. Propylamine, butyramine, putrescine, and cadaverine were the major amines formed. Amines constituted approximately 30% of total fermentation products in glucose-limited chemostats at a $D$ of 0.16/h but only about 5% in cultures grown at a $D$ of 0.04/h. This was partly due to the decrease in production of fermentation acids at the higher growth rate; however, lower levels of amines were produced in amino acid-limited chemostats, suggesting that glucose may repress amine formation in these cultures.

DISCUSSION

The major route through which bacteria produce amines is by decarboxylation of amino acids (13). Some species are thought to do this to maintain intracellular partial CO$_2$ pressure, but it is generally considered that the production of basic compounds provides a mechanism through which culture pH can be modulated during growth under acidic conditions (24).

Early studies by Gale (13) indicated that carbohydrate was required for amines to be produced and that they were formed during the later stages of bacterial growth in batch culture. Since many decarboxylases appear to be induced only under acidic conditions (24, 32), these conclusions may be based on the fact that a period of time was required for the products of fermentation to lower culture pH sufficiently for induction of enzyme synthesis to occur.

Our studies have shown that amines were produced by B. fragilis and C. perfringens in pH-controlled cultures during the active phase of growth (Fig. 1). They are in agreement with more recent work, which has indicated that under the appropriate growth conditions, bacteria can form amines during exponential growth (3).

In batch cultures of B. fragilis, significant quantities of amines (methylamine, dimethylamine, propylamine, pyrrolidine) were formed only under acid conditions (Table 1). The mechanisms of production of these amines were probably similar to those already documented in other bacteria (10). Dimethylamine, the major amine formed in batch culture by B. fragilis, is not produced by amino acid decarboxylation.

TABLE 3. Influence of glucose concentration and growth rate on the production of organic acids and amines by C. perfringens grown in continuous culture

<table>
<thead>
<tr>
<th>$D$ (per h)</th>
<th>Culture conditions</th>
<th>Organic acids</th>
<th>Amines</th>
<th>% Amines produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>L</td>
</tr>
<tr>
<td>0.04</td>
<td>Glucose limited</td>
<td>29.7</td>
<td>6.7</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Amino acid limited</td>
<td>44.6</td>
<td>10.7</td>
<td>31.9</td>
</tr>
<tr>
<td>0.08</td>
<td>Glucose limited</td>
<td>25.1</td>
<td>2.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Amino acid limited</td>
<td>17.1</td>
<td>2.4</td>
<td>5.7</td>
</tr>
<tr>
<td>0.16</td>
<td>Glucose limited</td>
<td>14.2</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Amino acid limited</td>
<td>11.3</td>
<td>1.8</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* Cultures were grown on a glucose-limited medium containing 5.0 g of peptone and glucose per liter and on an amino acid limited medium containing 5.0 g of peptone per liter and 5.0 g of glucose per liter.

** A, Acetate; B, butyrate; L, lactate; S, succinate; Me, methylvamine; Di, dimethylamine; Pr, propylamine; Py, pyrrolidine; Bu, butyramine; Pu, putrescine; Ca, cadaverine. ND, Not detected; T, trace (<0.1 mmol/g [dry weight] of cells)
but results from N-dealkylation of choline (10, 16). In contrast, methylamine and propylamine are produced by decarboxylation of glycine and \( \gamma \)-aminobutyric acid, respectively, whereas the cyclic secondary amine pyrrolidine is formed by oxidative deamination of putrescine, followed by ring closure (10).

The polyamines cadaverine and putrescine were important amine products of \textit{C. perfringens}. Cadaverine is formed in a number of bacteria by decarboxylation of lysine (32), and production of this amine was maximal at pH 6.0. In contrast, putrescine was principally formed at neutral pH. This amine is known to be the product of ornithine decarboxylation, and the decarboxylases involved in its production appear to operate at a higher pH than do other decarboxylases (32). The pH optimum for ornithine decarboxylase in \textit{Escherichia coli} is 6.9 (23), and production of the amine is maximal at pH 7.0 (9, 32). Putrescine was never detected in greater than trace amounts in cultures of \textit{B. fragilis} (Table 1), suggesting that it was converted to pyrrolidine as soon as it was formed.

Propylamine accounted for between 10 and 16% of the amines produced by \textit{C. perfringens} in batch culture (Table 1) and between 24 and 41% of the amines detected in continuous cultures (Table 3). Propylamine production by \textit{C. perfringens} was strongly influenced by pH and differed from \textit{B. fragilis} in that it was maximal at pH 7.0. Butylamine formed by decarboxylation of normvaline (10) was also pH dependent, occurring optimally at pH 7.0.

Carbohydrate was found to repress amine production in chemostat cultures of both organisms grown under pH-controlled conditions, although Gale noted that carbohydrate was required for optimal formation of amines by \textit{E. coli} and some clostridia (13). Total amine production increased with growth rate in the chemostat cultures of \textit{C. perfringens} and \textit{B. fragilis}, supporting results from the batch culture studies, which suggested that amine formation was growth related (Fig. 1).

Placing these results in the perspective of bacteria growing in the large intestine, colonic bacteroides would be expected to produce amines such as dimethylamine, propylamine, and pyrrolidine maximally under conditions of low pH and high growth rates found in the right large gut (7). Amine formation by \textit{C. perfringens} was more complex, however, in that some amines (dimethylamine, pyrrolidine, cadaverine) were produced mainly at acid pH, whereas others (methylamine, propylamine, butylamine, putrescine) were formed primarily at neutral pH (Table 1). Optimal production of these amines might therefore be expected to occur under the conditions of higher pH commonly found in the left colon (7).

The physiological significance of amine production in the large bowel remains to be established; however, a number of studies have suggested that the process may have potentially harmful effects for the host, particularly through the formation of \textit{N}-nitrosamines (29). These carcinogens have been detected in feces by many workers (12, 19), and several bacteria isolated from feces possess the metabolic capability to carry out \textit{N}-nitrosation of a variety of secondary amines (5, 30), including those found in cultures of \textit{B. fragilis} and \textit{C. perfringens}. The extent of nitrosamine formation in the gut depends on the availability of amines and nitrate-nitrite, as well as the local pH conditions. \textit{N}-Nitrosamine formation in vivo has been shown to occur in the presence of high dietary intakes of nitrate and amines (12, 33). The acid pH of the right colon, together with relatively high nitrate concentrations (27, 28) and elevated amine production, would thus potentiate \textit{N}-nitrosation (19, 30).

Amine production by colonic bacteria may affect the host in other ways. Amines are probably rapidly absorbed from the gut lumen in a manner similar to that of other small molecular species (22). Amines have been implicated in the etiology of hepatic coma (26) and psychological disorders, including migraine (2). Given the direct relationship between dietary protein intake and amine excretion observed in studies with infants (25) and adults (8), it is possible that some of the epidemiological findings relating dietary protein to increased incidence of colonic disease (16) may be explained by high levels of amine production by colonic bacteria.

LITERATURE CITED


