Growth of Enterobacter aerogenes in a Chemostat with Double Nutrient Limitations

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The behavior of Enterobacter aerogenes during growth in chemostats limited by single and double nutrient restrictions was examined. On the assumption that different essential nutrients act to limit growth in different ways, we selected pairs of nutrients likely to affect different aspects of metabolism. Results show that macromolecular cell composition can be controlled by using more than one nutrient restriction. The polysaccharide content of the cells is readily manipulated by the ratio of carbon to nitrogen in the inlet nutrients. Also, at low dilution rates, ratios of protein to ribonucleic acid are dependent on the ratio of phosphate to nitrogen in the input nutrients. An examination of both acetic acid and metabolite production (as measured by ultraviolet absorbance of culture filtrates) showed that accumulation of these products was dependent on both dilution rate and type of nutrient limitation(s). These results were examined in terms of the problems of translation of batch to continuous culture processes and the use of selected nutrient limitations to control noncellular product formation.

The cellular composition and biochemical activities of microorganisms are functions of the growth environment. Investigations (2, 9) have shown that in continuous culture the macromolecular composition of Enterobacter aerogenes and other organisms varies with the dilution rate. The various intracellular metabolite pools also change with growth rate (1). These physiological and biochemical properties are dependent on the pH, temperature, and ionic composition of the environment. Consequently, manipulating the environment of the cell gives the investigator considerable control over the metabolic activities of growing and resting microorganisms. Many studies have been conducted to elucidate the relationships between growth environment and cellular physiology in continuous culture (10), which provides a tool for maintaining time-invariant or steady-state growth in a constant, controllable environment. In previous studies using the chemostat form of continuous culture (6), growth could be limited by restricting any single essential nutrient. By examining possible limiting nutrients in a minimally defined medium, a hypothesis has been formulated concerning the mode of limitation exerted by each nutrient (Table 1).

Previous work (2, 8) has established the degree to which one can control cell composition under the influence of single nutrient limitations. The results of these studies have raised the question of whether it is possible to grow cells under multiple nutrient limitations to achieve even greater control over cellular processes. The concept of a multiple nutrient limitation implies that the growth and function of a cell are regulated, or otherwise affected, by the limited availability of more than one nutrient. It is likely that both cell composition and the ability of cells to synthesize noncellular metabolic products depend on the availability of more than one nutrient. Our hypothesized modes of action for various nutrient limitations suggested that multiple nutrient limitations were possible. To explore this question experimentally, we examined the growth of E. aerogenes NCTC 418 in a chemostat with variable ratios of carbon to nitrogen and nitrogen to phosphate in the inlet medium. Biochemical activities of the culture were assessed by measuring macromolecular cell composition, excretion of metabolic products, and residual levels of essential nutrients.

MATERIALS AND METHODS

Organism and growth media. E. aerogenes NCTC 418 was obtained from D. W. Tempest and maintained by monthly transfers on tryptone glucose extract agar (Difco) slants. The organism was grown...
at 37 C and pH 6.5. Table 2 gives the composition of the growth media for carbon-limited and/or nitrogen-limited and/or phosphate-limited chemostats.

Analytical procedures. The biuret reaction (11) was used to measure cell protein; bovine serum albumin (Pentex) provided a standard. The total carbohydrate content of the cells was measured by the anthrone reaction (3), using glucose as a standard. Cellular ribonucleic acid (RNA) was extracted by the Schmidt-Tannhauser procedure as described by Munro and Fleck (8). Absorbance of the extract was measured at 260 nm, and the RNA was quantitated with an extinction coefficient of 32 liters/g per cm (4). RNA from E. coli (Calbiochem) provided a standard.

Glucose in the culture supernatant was measured with glucose oxidase (Worthington Biochemicals). Ammonia was measured by the alkaline-phenol reaction (7) on an autoanalyzer (Technicon). Acetate in the supernatant was determined by the gas chromatography procedure described by P. S. Masurekar (MS thesis, Massachusetts Institute of Technology, Cambridge, 1968). Phosphate was measured by the procedure of Sumner (12). Ultraviolet-absorbing material in the culture supernatant was quantitated by light absorption at 260 nm, and the values obtained were divided by the cell dry weight to normalize differences in cell concentrations.

The results of all the analyses of the cells and culture supernatants from steady-state cultures represent the average values of at least two different cell samples collected on different days, except where noted. Steady states were initially established on the basis of constant culture turbidity and later verified by determinations of the composition of the cell-free supernatant and cell dry weights.

Experimental apparatus. The continuous culture apparatus used in this work was described in detail by C. L. Cooney (Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1970). The 1-liter vessel with four Teflon baffles contained 400 ml of medium, which was agitated by a magnetic stirrer. Sterile air was sparged directly over the stirring bar at 1 liter/min. The pH control system incorporated an Ingold combination pH electrode (Chem- apex, Inc.) in conjunction with a Leeds and Northrop pH controller, which actuated a pump that added 1.0 M NaOH on demand. Medium was pumped from a 16-liter polypropylene carboy with a variable-speed Cole-Parmer Masterflex pump, and an overflow tube maintained a constant volume in the vessel. The entire vessel was set in a water bath controlled at 37 C.

**RESULTS**

Cell macromolecular composition. The macromolecular composition of *E. aerogenes* NCTC 418 was measured in chemostats limited singly by carbon, nitrogen, or phosphate and simultaneously by nitrogen and carbon or nitrogen and phosphate (Tables 3 and 4).

For each limiting nutrient tested, the RNA content increased with increasing dilution rate (Fig. 1). The protein content also increased with the dilution rate, but to a much lesser degree than the RNA; therefore, the ratio of protein to RNA decreased as the dilution rate increased (Fig. 2). Interestingly, at a dilution rate of 0.25/h, the ratio of protein to RNA was nearly identical for cells grown under nitrogen or carbon limitations. However, cells in a phosphate-limited environment exhibited a marked increase (about 40%) in this ratio. This disparity lessened as the dilution rate increased. The difference in the ratios of protein to RNA for the various nutrient limitations was statistically significant at the low dilution rate but not at the higher dilution rate.

Although these implications will be consid-
TABLE 3. Macromolecular composition of E. aerogenes grown in carbon- and/or nitrogen-limited chemostats

<table>
<thead>
<tr>
<th>Limiting nutrient</th>
<th>Dilution rate (1/h)</th>
<th>Protein (%)</th>
<th>RNA (%)</th>
<th>Carbohydrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon⁰</td>
<td>0.22</td>
<td>69</td>
<td>13</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>63</td>
<td>18</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.77ᵇ</td>
<td>72</td>
<td>25</td>
<td>8.7</td>
</tr>
<tr>
<td>Nitrogen⁰ (ammonia)</td>
<td>0.24</td>
<td>53</td>
<td>11</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>52</td>
<td>17</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>58</td>
<td>21</td>
<td>9.5</td>
</tr>
<tr>
<td>Dual⁰ (nitrogen and carbon)</td>
<td>0.27</td>
<td>60</td>
<td>12</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>60</td>
<td>17</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>0.78ᵇ</td>
<td>68</td>
<td>21</td>
<td>6.8</td>
</tr>
</tbody>
</table>

⁰ Inlet concentrations (mg/liter): glucose, 500; ammonia, 40.
ᵇ Values were obtained for cultures at only one steady state.
ᶜ Inlet concentrations (mg/liter): ammonia, 40; glucose, 1,000.
ᵈ Inlet concentrations (mg/liter): ammonia, 40; glucose, 640.

TABLE 4. Macromolecular composition of E. aerogenes grown in phosphate- and/or nitrogen-limited chemostats

<table>
<thead>
<tr>
<th>Limiting nutrient</th>
<th>Dilution rate (1/h)</th>
<th>Protein (%)</th>
<th>RNA (%)</th>
<th>Carbohydrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen⁰ (ammonia)</td>
<td>0.25</td>
<td>53</td>
<td>9.8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>58</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>60</td>
<td>20</td>
<td>7.2</td>
</tr>
<tr>
<td>Phosphate⁰</td>
<td>0.25</td>
<td>72</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>67</td>
<td>17</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>56</td>
<td>19</td>
<td>5.7</td>
</tr>
<tr>
<td>Dual⁰ (nitrogen and phosphate)</td>
<td>0.25</td>
<td>53</td>
<td>9.7</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>61</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>71</td>
<td>19</td>
<td>9.1</td>
</tr>
</tbody>
</table>

⁰ Inlet concentration (mg/liter): glucose, 1,000.
ᵇ Inlet concentrations (mg/liter): ammonia, 43; phosphate, 32.1.
ᶜ Inlet concentrations (mg/liter): phosphate, 23; ammonia, 125.
ᵈ Inlet concentrations (mg/liter): ammonia 47; phosphate, 21.

Table 3 shows the macromolecular composition of cells grown under a simultaneous limitation of glucose and nitrogen and under a limitation of each individually. To achieve the double nutrient limitation, the chemostat was first operated under a carbon limitation, with input glucose and ammonia initially set at 500 mg/liter and 40 mg/liter, respectively. Effluent ammonia concentration under these conditions was approximately 6 mg/liter (Table 5).
concentration is at least 60 times the $K_v$ value for ammonia; thus the nitrogen source greatly exceeded cell requirements. Consequently, estimates of the macromolecular composition of cells taken from this system represent values for bacteria grown under carbon-limited conditions. The glucose input was increased to 640 mg/liter, whereas the ammonia input remained constant at 40 mg/liter. The incremental increase of 140 mg/liter in the input glucose concentration was determined by calculating the amount of carbon source that the cells would require to incorporate the remaining 6 mg of extracellular ammonia per liter into cell mass. Glucose yield was calculated to be 0.5 g of cell/g of glucose, and nitrogen yield was assumed to be 10 g of cell/g of ammonia. This second set of nutrient conditions was designed to exert simultaneous nitrogen and glucose limitations. The conclusion that the culture was nitrogen- as well as carbon-limited was based on the following observations: (i) both the effluent glucose and ammonia concentrations were very low (Table 5); (ii) a further increase in glucose did not proportionately increase cell mass; (iii) total cell carbohydrate at $D = 0.27/h$ was higher than that found under glucose-limiting conditions alone (Table 3) (these cells accumulate glycogen when starved for nitrogen in the
presence of excess carbon source [5]); and (iv) the cells excreted about the same amount of acetate as those in a strictly carbon-limited culture (Fig. 4).

After this series of steady states, the glucose input was increased to 1,000 mg/liter to create a nitrogen-limited chemostat. The result was an increase in carbohydrate at low dilution rates (Table 3) and a further increase in acetate excretion (Fig. 4). When cellular protein and RNA were compared at a constant dilution rate for increasing concentration ratios of glucose to ammonia in the inflowing medium, the absolute concentration of both cell components generally tended to decrease. This phenomenon was most noticeable at low growth rates. However, the ratio of protein to RNA was relatively constant (Fig. 2) when compared at a given dilution rate.

The procedure used to examine the influence of phosphate on the composition of cells from a nitrogen-limited chemostat (Table 4) was similar to the one used to study cells from carbon-limited and/or nitrogen-limited systems. Table 4 lists values for cells from an ammonia-limited chemostat that have a low, but not limiting, concentration of phosphate. In this case, the effluent phosphate concentration varied between 8.4 and 11.1 mg/liter, depending on the dilution rate (Table 6). These effluent phosphate concentrations are at least 10 times the $K_v$ value. The ammonia-limited cultures (cell compositions presented in Tables 3 and 4) were grown under slightly different conditions. In the first case, the inlet phosphate was greater than 3,000 mg/liter, and in the second it was 32.1 mg/liter. Despite this difference in medium compositions, the values are not strikingly different.

The inlet phosphate concentration was lowered to 21 mg/liter, whereas the inlet ammonia concentration remained constant (Table 4). Macromolecular compositions were similar for cells from ammonia-limited cultures with excessive or restricted phosphate. A slightly increased ratio of protein to RNA was noted at dilution rates of 0.5 and 0.75/h for cells from cultures limited by ammonia and restricted by phosphate compared with values for cells from cultures limited by ammonia alone.

When the ammonia inlet concentration was increased to permit phosphate limitation only, the ratio of protein to RNA in the cells increased dramatically at $D = 0.25/h$. At higher dilution rates, the ratio returned to values similar to those observed under ammonia limitation.

The evidence for a dual limitation of ammonia and phosphate includes the following: (i) the effluent concentrations of both nutrients were at limiting values (Table 6); and (ii) the

![Fig. 4. Acetic acid excretion by E. aerogenes grown in single and dual nutrient-limited chemostats. Symbols: ○, nitrogen limited; +, nitrogen and phosphate (23 mg/liter) limited; △, nitrogen and phosphate (32.1 mg/liter) limited; ○, nitrogen limited; □, carbon limited.](image)

**Table 6. Effluent concentrations of essential nutrients in cultures of E. aerogenes in nitrogen- and/or phosphate-limited chemostats**

<table>
<thead>
<tr>
<th>Limiting nutrient</th>
<th>Dilution rate (1/h)</th>
<th>Ammonia (mg/liter)</th>
<th>Phosphate (mg/liter)</th>
<th>Glucose (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen$^b$ (ammonia)</td>
<td>0.25 0.50 0.74</td>
<td>0.50 0.74 0.52</td>
<td>0.70 0.70 0.70</td>
<td>9.6 8.4 0.52</td>
</tr>
<tr>
<td>Phosphate$^c$</td>
<td>0.26 0.52 0.70</td>
<td>67 79 90</td>
<td>0.54 1.2 0.6</td>
<td>11.1 1.5 9.9</td>
</tr>
<tr>
<td>Dual$^d$ (nitrogen and phosphate)</td>
<td>0.25 0.50 0.75</td>
<td>0.1 0.2 0.2</td>
<td>0.54 0.6 0.33</td>
<td>1.4 0.8 0.33</td>
</tr>
</tbody>
</table>

$^a$ Inlet concentration (mg/liter): glucose, 1,000.
$^b$ Inlet concentrations (mg/liter): ammonia, 43; phosphate 32.1.
$^c$ Inlet concentrations (mg/liter): phosphate, 23; ammonia, 125.
$^d$ Inlet concentrations (mg/liter): ammonia, 47; phosphate, 21.
patterns of acetate excretion at $D = 0.25$ and 0.5/h fell between the values for a single ammonia or phosphate limitation.

**Formation of products under different nutrient limitations. (i) Essential nutrients.** Effluent concentrations of several essential nutrients, including the limiting nutrient(s), were measured at each steady state. Table 5 gives results for assays of culture supernatants under carbon and/or nitrogen limitations, and Table 6 gives results for those under nitrogen and/or phosphate limitations. The quantity of effluent glucose is not shown for those samples in which there was interference with the glucose oxidase procedure. When interference occurred, samples tested for glucose gave a purple-gray product instead of the normal brown-orange of the oxidized chromogen (3,3′-dimethoxybenzidine). The degree of interference was a function of both dilution rate and limiting substrate, and the reaction generally occurred at $D = 0.75$/h for all nitrogen-limited conditions.

The effluent glucose concentration under carbon limitation (Table 5) fits the Monod growth kinetic model quite well and yields a $K_v$ value for glucose of 1.0 mg/liter. For all growth conditions, effluent glucose approached a very low value near its $K_v$ concentration. The effluent ammonia concentration under nitrogen limitation was so low that it was difficult to measure. The alkaline-phenol procedure used in conjunction with a Technicon autoanalyzer has a lower sensitivity limit of about 0.1 µg/ml.

Unlike glucose, neither ammonia nor phosphate were completely removed from the medium when they were not the primary or secondary limiting nutrient.

(ii) Catabolite excretion. The production of catabolic intermediates from glucose indicated inefficient utilization of the carbon-energy source. Acetic acid excretion varied markedly with changes in the nutrient composition of the media during growth (Fig. 4). At a given dilution rate acetic acid excretion was minimal in carbon-limited cultures. Under nitrogen limitation, the amount of acetic acid excreted was proportional to the amount of excess glucose available to the cell (compare the slopes of the curves for nitrogen-limited cultures with those for nitrogen- and carbon-limited cultures [Fig. 4]). Furthermore, the inlet concentration of phosphate affected acetate production (Fig. 4). Thus, the availability of at least three essential nutrients (glucose, ammonia, and phosphate) affected the production of acetic acid.

(iii) Metabolite excretion. The third class of compounds examined in the extracellular environment was metabolites that did not appear to be direct products of glucose catabolism. These materials were characterized by their light absorption at 260 nm; the amounts excreted into the medium varied over a fivefold range under the growth conditions investigated but were greatest in nitrogen-limited cultures (Fig. 5). Although the relative positions change with dilution rate, excretion generally decreased when the quantity of carbon or phosphate was lowered to make it a second limiting nutrient (Fig. 5). A double limitation of nitrogen and phosphate suppressed excretion more than a limitation of phosphate alone, and a carbon shortage produced the greatest suppression.

Much of the excreted material may have been nucleotides and/or nucleosides, judging from the positive orcinol reaction obtained. Since orcinol reacts not only with ribose, but also to a limited extent with hexoses, and possibly other interfering materials, the presence of nucleic acid components was not unequivocally established by this reaction. Anion-ex-
change chromatography of culture supernatant fluids showed four to five major components in the medium from phosphate- or ammonia-limited cultures, but these did not correspond with any of the common nucleotides (C. L. Cooney, Ph.D. thesis). Thus, the identity of the extracellular, ultraviolet-absorbing products remains unknown. Interestingly, ion-exchange chromatograms showed that growth of the bacteria under nitrogen and phosphate limitations led to excretion of different products.

DISCUSSION

When carbon or nitrogen alone limited growth, the ratio of protein to RNA was a function of only the dilution rate (and not of the ratio of input carbon to nitrogen); the ratio of protein to RNA decreased as the dilution rate increased. This decrease was primarily the result of an increased level of RNA while the protein concentration remained relatively constant. This behavior pattern may be explained by the need of the cells to synthesize protein faster as their growth rate increased.

When phosphate was the growth-limiting nutrient, the ratio of protein to RNA was higher at low dilution rates compared with the ratio resulting from carbon- or nitrogen-limited growth. The increased ratio of protein to RNA was interpreted here as being consistent with the hypothesis that a phosphate limitation restricts nucleic acid synthesis. Under a phosphate limitation the cell would have a minimal quantity of nucleic acid, which is predominately RNA.

When a double limitation of carbon and nitrogen was imposed, the macromolecular composition fell between the limits established by single nutrient limitations. Consequently, it became possible to design a medium that would produce cells with a range of compositions between the limits established by the single nutrient limitations. The major constraint appeared to be the ratio of protein to RNA, which was determined by the dilution rate under carbon and nitrogen limitations. However, the limits of variation in composition were somewhat more flexible when cells were cultured with a phosphate limitation, because at low growth rates the ratio of protein to RNA was not only a function of the dilution rates, but also of the nature of the growth limitation.

One major variable influencing cell composition is the concentration of storage materials, such as polysaccharide, lipid, or poly-β-hydroxybutyric acid. When these materials accumulate, they dilute the relative proportions of other cell components. Such carbon-energy reserves are not uncommon in cells grown under nitrogen-limited conditions (5). Although Tempest and Dicks (14) found that E. aerogenes did not accumulate carbon reserve material under phosphate-limited conditions, our findings indicate that intracellular carbohydrates accumulated to comprise 15 to 20% of the dry cell weight, but only at dilution rates less than 0.50/h. This discrepancy might be explained by a change in the nature of the growth-limiting nutrient at low growth rates. Most of the cellular phosphate was in the RNA fraction, and the RNA level was directly proportional to the growth rate. At low growth or dilution rates, the demand of the cell for phosphate decreased, and a given amount of phosphate was able to support a larger cell population. Therefore, in the cultures incubated at a dilution rate of 0.25/h, the cells might have been nitrogen-limited and/or phosphate-limited, even though at higher dilution rates on the same medium they were phosphate-limited.

In addition to differences in cell composition under double-nutrient limitations, components of the extracellular environment varied with the type of nutrient limitation. When glucose was the sole limiting nutrient, the cells responded according to the Monod model; yet when other nutrients limited growth, glucose was still maintained in low (i.e., near $K_0$) concentrations, even though it was supplied in considerable excess. Then, under ammonia and/or phosphate limitation(s), the cells were still readily able to assimilate glucose and to form cell carbohydrate, catabolic products of glucose (such as acetic acid), and a variety of ultraviolet-absorbing excretion products. Therefore, it may be possible to choose a limiting nutrient that restricts cell growth yet permits the carbon source to be shunted through pathways for oversynthesis of desired products.

By knowing how cultural conditions affect the kinetics of growth and product formation, one may be able to design conditions for maintaining, and possibly enhancing, product formation. In this respect, the imposition of a dual nutrient restriction on cell metabolism may be valuable in achieving a tighter control of useful metabolic pathways.

LITERATURE CITED