Effect of Growth Rate on Histidine Catabolism and Histidase Synthesis in *Aerobacter aerogenes*

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A study was made of how the catabolism of a carbon and energy source is affected by the biosynthetic demands of growing bacterial cells. Cultures of *Aerobacter aerogenes* in L-histidine medium were grown in a chemostat at rates determined by the supply of either sulfate or a required amino acid, L-arginine. It was discovered that the rate at which these cells grow under a biosynthetic restriction determines both the rate and the pattern of histidine degradation. (i) Histidine catabolism is partially coupled to the growth rate. This coupling is achieved by catabolite repression of histidase (histidine ammonia lyase; EC 4.3.1.3.), and also by a slightly decreased in vivo function of this enzyme at low growth rates. (ii) The looseness of the coupling results in a direct relationship between growth rate and growth yield, and possibly is correlated with an altered pattern of carbon flow from histidine. (iii) Sudden decreases in growth rate cause total repression of histidase synthesis for substantial periods of time. (iv) Sudden release of biosynthetic restriction leads rapidly to an increase in the functioning of the cells' complement of histidase, an increase in the rate of synthesis of this enzyme, and an increase in the growth yield from histidine.

When growing bacterial cells are harvested and suspended in a nongrowth medium, they continue to metabolize their energy-yielding substrate at an appreciable rate. This common observation leads naturally to the view that the coupling between catabolism and anabolism in these cells is a very loose one.

Gunsalus and Shuster (2) and Senez (16) have reviewed this notion and have cited other lines of evidence that catabolism is not restricted by the rate of cellular synthesis. Not only can resting cells metabolize substrates vigorously, but there is also evidence that, during certain conditions of balanced growth, catabolism can proceed at a rate disproportionate to growth. In *Desulfovibrio desulfuricans*, substitution of molecular nitrogen for ammonia as the nitrogen source causes a marked reduction in growth rate with no parallel reduction in the rate of degradation of the major carbon and energy source (5). Similarly, substitution of nitrate for ammonia in *Aerobacter aerogenes* has been reported to reduce the growth rate without a parallel decrease in catabolism (13). Rosenberger and Elsden (14) grew *Streptococcus faecalis* anaerobically in a rich medium in a chemostat with tryptophan limiting the growth. They found that the rate of substrate consumption per unit weight of cells was constant at all growth rates. More recently, another possible instance of uncoupling between catabolism and growth has been seen in *Clostridium pasteurianum* in which sucrose consumption fails to be diminished when the growth rate is lowered by restricting the supply of NH₃ (1).

At first glance, there would seem to be no difficulty in accepting this view, and the absence of controls to integrate catabolism with biosynthesis may appear of little consequence. After some reflection, however, this view must be modified. First, it stands in sharp contrast to the fineness with which the bacterial cell adjusts the rates of individual biosynthetic pathways and integrates them with the polymerization reactions of growth. Second, substrate degradation in the face of restricted growth requires that adenosine diphosphate (ADP), nicotinamide adenine dinucleotide, and other cofactors be continuously regenerated. This regeneration must be carefully controlled so that adenosine triphosphate (ATP) and reduced cofactors are not similarly squandered when conditions permit rapid growth.

1 These studies are taken from a thesis submitted by D. E. J. in partial fulfillment of the requirements for a Ph.D. degree at Purdue University, 1966. Part of this work was presented at the Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, 1963.

2 Present address: Miami Valley Laboratories, Proctor and Gamble Co., Cincinnati, Ohio 45239.
other words, an “uncontrolled” rate of substrate consumption would be possible only if certain other catabolic processes were extremely sensitively controlled.

Finally, there is a third cause for concern. The theory of catabolite repression (8) rests on the premise that the cell does make a regulatory response to an imbalance between catabolism and biosynthesis. Any substrate which is degraded by inducible, catabolite-repressible enzymes should, during steady-state growth, be metabolized at a rate which is commensurate with the growth rate of the cells, for catabolite repression should restrict the synthesis of the appropriate catabolic enzymes. Thus, when the growth rate of a culture is reduced by limiting the rate of supply of some growth factor, continued rapid substrate degradation will lead to high intracellular levels of repressing metabolites. Adjustments in cellular metabolism must occur to lower the levels of these metabolites during subsequent slow growth. Such adjustments could conceivably be of two sorts: (i) a reduction in the rate of substrate degradation to couple it to cell growth rate by either lowered synthesis or lowered activity of the catabolic enzymes; or (ii) an expansion (by enzyme synthesis) of some metabolic route to prevent the accumulation or to dispose of excess metabolites.

In view of these implications, we have explored the metabolism of L-histidine by *Aerogenes* (9). The cells grow sufficiently rapidly in minimal medium on this substrate to permit their growth rate to be manipulated over an eightfold range in a chemostat. The principal finding in our study is that L-histidine metabolism is affected by the growth rate of the cells.

A summary of some preliminary experiments in this study has appeared (11).

MATERIALS AND METHODS

Organism. All experiments were performed with *Aerogenes* LC1. This strain was derived from strain XXXY, the wild strain used in this laboratory, by sequential selection for two blocks in the L-arginine biosynthetic pathway, one before and one after L-citrulline. The double block enabled continuous cultures to be maintained on limiting L-arginine for periods as long as 1 week without overgrowth of revertant cells.

Media. A minimal medium was used in all experiments; it consisted of basal salt solution P (Na₂HPO₄·7H₂O, 1.34%; KH₂PO₄, 1.36%; CaCl₂, 0.0011%; MgSO₄·7H₂O, 0.0246%; all w/v) supplemented with (NH₄)₂SO₄ (0.2%, w/v) and with L-histidine as the major carbon and energy source at concentrations that depended on the particular experiment. The growth factor, L-arginine, was added to give a concentration of 40 µg per ml in media to be used for batch culture or for sulfate-limited cultures, and 6 to 9 µg per ml in media to be used for L-arginine-limited chemostat cultures. To construct a sulfate-limited medium, the MgSO₄ and (NH₄)₂SO₄ of the minimal medium were replaced by an equivalent amount of MgCl₂ and NH₄Cl, respectively, and sufficient (NH₄)₂SO₄ was added to give a sulfur concentration of 1.6 µg per ml.

Growth measurements. Batch cultures were grown aerobically in Erlenmeyer flasks filled to one-tenth their capacity. They were agitated on a rotary-action shaker at 37 C. Growth was followed by measuring the optical density of the culture at 420 nm in a Zeiss spectrophotometer, PMQII. Growth rates were expressed as the specific growth rate constant, k, calculated by the relation:

\[
k = \frac{\ln C_2 - \ln C_1}{t_2 - t_1}
\]

where C₁ and C₂ are the optical densities of the culture at times t₁ and t₂, respectively, and t is expressed in hours. An optical density of 1.00 at 420 nm with a 1-cm light path corresponded to 80 µg of protein and 150 µg of bacterial dry weight per ml.

Chemostat cultures were grown at 37 C in glass chemostats of 50-, 150-, or 250-ml growth chamber capacity. The apparatus is described in detail elsewhere (D. Jensen, Ph.D. Thesis, Purdue Univ., Lafayette, Ind., 1966). In essence, it consists of a water-jacketed growth chamber with a constant liquid volume maintained by an overflow tube; mixing and aeration are provided by a rapid stream of warmed moist air bubbling through a sintered-glass sparger at the bottom of the growth chamber; the steady addition of fresh medium is accomplished by a finger pump operating on a tube from the medium reservoir. Flow rates were constant to within 3% over many days of operation. Cell density varied with growth rate within the small limits expected theoretically and was constant at any given growth rate to within 3%. Temperature variation in the growth chamber was less than 0.1 degree C. Measurement of the ribonucleic acid (RNA)-protein ratio of the cells at various flow rates gave values indicative of the corresponding growth rate of the organism. During steady-state operation, the specific growth rate constant, k, of a chemostat culture is simply equal to the dilution rate, d,

\[
d = \frac{k \cdot V}{d}
\]

where v is the flow rate in milliliters per hour and V is the volume of the growth chamber in milliliters. During steady-state operation, there is no change in the optical density of the effluent culture. Should the cells grow, from time t₁ to time t₂, at a rate which is faster than the dilution rate, the optical density will increase; conversely, should the cells be unable to grow as fast as the contents of the growth chamber are diluted, the optical density will fall. To a close approximation, the specific growth rate during such a
period of non-steady-state operation can be estimated from the following equation:

\[ k = d + \frac{\ln C_2 - \ln C_1}{t_2 - t_1} \]  

(3)

where, as before, \( d \) is the dilution rate in hr⁻¹, and \( C_1 \) and \( C_2 \) are the culture densities of the culture at times \( t_1 \) and \( t_2 \), respectively, expressed in hours.

**Measurement of cellular consumption of L-histidine.** Samples were removed from batch cultures at specified times and quickly brought to 0 C. Cells and medium were separated by centrifugation at 12,000 × g for 10 min. If necessary, the supernatant medium was stored at -20 C. The amount of L-histidine was assayed as described below. The amount of L-histidine (in milligrams per milliliter) removed from the medium was then plotted against the cell dry weight (milligrams per milliliter) formed. During balanced growth, this plot yields a straight line, the slope of which is the milligrams of L-histidine used to produce 1 mg of cell dry weight. This value can be converted to the specific rate of L-histidine metabolism by multiplying by \( k \) to yield milligrams of L-histidine consumed per hour by 1 mg of cells.

Similarly, L-histidine consumption was measured in steady-state chemostat cultures. Samples of effluent culture were collected in tubes held at 0 C, and cells and medium were separated by centrifugation. Portions of the medium were assayed for L-histidine, and the specific rate of L-histidine consumption was calculated by the following equation:

\[ V_B = \frac{(h_{in} - h_{out})v}{m} \]  

(4)

where \( V_B \) is the rate of L-histidine consumption (milligrams of L-histidine per hour per milligram of cells), \( h_{in} \) and \( h_{out} \) represent the steady-state concentration of L-histidine (milligrams per milliliter) in the input medium and the outgoing culture, respectively, \( v \) is the flow rate (milliliters per hour), and \( m \) is the total dry weight (milligrams) of cells in the growth chamber. Since \( m \) is equal to the total volume of the growth chamber, \( V \) (milliliters), multiplied by the concentration of cells, \( C \) (mg per ml), equation 4 can be written as

\[ V_B = \frac{(h_{in} - h_{out})v}{CV} = \frac{(h_{in} - h_{out})k}{C} \]  

(5)

provided that steady-state conditions have been achieved.

**Preparation of extracts.** Samples (15 to 25 ml) of cultures containing approximately 100 μg of total protein per ml were collected at 0 C. The cells were harvested by centrifugation at 0 C, washed twice with sodium pyrophosphate buffer (10⁻² M, pH 9.2) and finally suspended in 2 ml of the same buffer. The cells were then disrupted by sonic treatment for 2 min with a 20-kv Sonifier (Branson Instruments, Inc., Stanford, Conn.), and the resulting mixture was centrifuged at 12,000 × g for 20 min. The supernatant liquid was decanted and assayed immediately for total protein and for enzyme activity.

**Enzyme assay.** Histidine ammonia lyase (EC 4.3.1.3), commonly called histidase, was assayed by measuring the formation of uracanone from L-histidine. Reaction mixtures (3 ml) contained sodium pyrophosphate (10⁻² M, pH 9.2) and crude extract (50 to 150 μg of protein). The reaction was run at 25 C, and was initiated by the addition of L-histidine. The course of the reaction was followed by monitoring the increase in absorbance at 277 nm in a recording spectrophotometer (Gilford Instruments, Oberlin, Ohio). One unit of histidase was defined as that activity which converts 1 μmole of L-histidine to uracanone per hr under the conditions of the assay, assuming that an increase in absorbance of 1.0 was equivalent to the formation of 5.55 × 10⁻³ μmoles of uracanone per ml.

**Differential rate of enzyme formation.** During steady-state growth, the differential rate of formation of any enzyme is numerically equal to its specific activity (total units per milligram of protein), whether the culture is unrestricted or growing in a chemostat. In some chemostat experiments, it was desirable to estimate the differential rate of histidase formation during non-steady-state conditions. This estimation was made by first using equation 3 to calculate the overall growth rate \( k \) of the culture between \( t_1 \) and \( t_2 \), the time interval in question. Next, the growth increment, \( C_2/C_1 \), of a hypothetical, equivalent batch culture was calculated from the relationship given in equation 1: \[ \ln C_2/C_1 = k(t_2 - t_1) \]  

Knowing the specific activity (units per milligram of protein) of histidase at \( t_1 \) and \( t_2 \), it was then possible to calculate the amount of enzyme, \( e_1 \) and \( e_2 \), associated with the total protein at \( t_1 \) and \( t_2 \), respectively, in the hypothetical batch culture. A plot of \( e \) against \( C \) then enabled one to compare the slope of this function between \( C_1 \) and \( C_2 \) with the known (steady-state) slope between the origin and \( C_1 \).

**Chemical determination.** L-Histidine was assayed by the method of Jorpes (4) in which diazotized sulfanilic acid reacts with the imidazole group of L-histidine to form a colored product.

Protein was measured by the method of Folin (7) and RNA by the orcinol method (15).

**Manometric analysis.** In one experiment, the amounts of CO₂ evolved and O₂ taken up by resting cells were measured by standard manometric techniques (17). Details are listed in the Results section.

**Metabolism of 14C-L-histidine.** To trace the fate of L-histidine carbon during growth at different biosynthetically restricted rates (results shown in Tables 3 and 4), the chemostat apparatus was modified by adding tubing to the exit port so that the effluent gas (air that had bubbled through the culture in the growth chamber) was led through two consecutive 2-liter flasks half full of 10% KOH to trap CO₂. A T tube in this line adjacent to the exit from the growth chamber permitted the collection of effluent culture. For radioactivity measurements of CO₂ produced by the culture, the effluent gas was passed through a KOH solution (5 N) for either 0.5 or 1.0 hr. The chemostat was run with sulfate limitation,
first at \( k = 0.210 \), and later at \( k = 0.714 \). As soon as steady-state growth was reached at each flow rate, 10 gas samples and 10 culture samples were taken. The CO\(_2\) in the gas samples was precipitated by the addition of BaCl\(_2\), and the precipitate was collected on membrane filters. One portion of the culture was filtered to permit the separate counting of cells and filtrate. The radioactivity in a portion of the unfiltered culture was determined separately. The filtered medium was assayed colorimetrically for L-histidine. Separate samples were taken to assay the cells for histidase. All samples were counted for 10 min in 15 ml of the scintillation fluid described by Marr et al. (10), by use of a Packard scintillation counter, and in all cases multiple samples of different sizes were measured to detect any self-absorption.

To measure the distribution of L-histidine carbon in resting-cell suspensions (results appearing in Tables 3 and 4), cells were prepared by growth in a sulfur-restricted chemostat \((k = 0.19)\) and by growth in unrestricted batch culture \((k = 0.85)\). Both populations of cells were centrifuged, washed twice, and suspended in 42 ml of basal medium lacking both sulfur and L-arginine, and containing \(^{14}\text{C-}L\)-histidine \((1.50 \text{ mg/ml} ; 2.9 \mu\text{c per mmole})\). The suspensions were placed in separate closed glass chambers fitted with a center well containing 10 ml of KOH \((10\%, \text{ w/v})\). They were incubated at 37 C for 8 hr (slow cells) or 2.5 hr (fast cells). Duplicate samples of each incubation mixture were removed at the start and end of the experiment to establish the optical density, radioactivity, histidine content, RNA-protein ratio, and histidase level of the cultures. At the end of the incubation, 1 ml of the KOH solution was placed into 20 ml of 1.0 m BaCl\(_2\) to precipitate BaCO\(_3\). Samples of cells on membrane filters, filtered medium, and BaCO\(_3\) precipitates were counted in a scintillation counter as described above. In all cases, multiple samples of different sizes \((e.g., 0.25 \text{ and } 0.50 \text{ ml of filtered medium})\) were counted to detect self-absorption.

**RESULTS**

**Rate of L-histidine metabolism during steady-state growth.** In a large number of separate experiments, cultures of *A. aerogenes* LCI were grown in the chemostat at 37 C with either sulfate or L-arginine limiting. In each experiment, the culture was maintained at a particular growth rate for many generations to ensure that steady state growth had been established. Chemical assay to determine the concentration of L-histidine in the incoming medium and the outflowing culture \((\text{after removal of the cells})\) was performed. Samples were collected at intervals throughout a long time period until a steady-state level of histidine concentration had been reached. The cell density and the flow rate of the chemostat were similarly monitored. It was then possible to calculate the specific rate of histidine disappearance in the manner described under Materials and Methods.

Some representative data from these studies are presented in Table 1 to show the actual magnitude of the measured parameters and to clarify the calculation of the specific rate of histidine catabolism. A summary of all of the measurements is presented in Fig. 1, in which the specific rate of L-histidine catabolism is plotted as a function of growth rate for sulfur-limited and for L-arginine-limited cultures. Data from three experiments with batch (unrestricted) cultures are shown in the same figure.

The results provide a clear demonstration that the rate of L-histidine catabolism in these cells is set at different levels at different growth rates. There appears to be a nearly linear relationship, indicating that catabolism of L-histidine is approximately 80% coupled to the biosynthetic demands of the cells over the whole range of possible growth rates, whether sulfate or L-arginine is the rate-limiting nutrient.

**Level of histidase during steady-state growth.** The partial coupling of histidine catabolism to growth rate raises the question of what restricts substrate degradation during biosynthetically restricted growth. It was reasoned that catabolite repression should be responsible for this coupling, and so the level of the first enzyme in the inducible series that degrades L-histidine was assayed in cells grown in the chemostat at different growth rates with either sulfate or L-arginine restriction.

Cells of strain LCI from chemostat cultures at different growth rates were harvested and assayed for histidase as described in Materials and Methods. At each flow rate, growth was continued until steady-state conditions had been achieved. The measured levels of histidase are plotted as a function of growth rate in Fig. 2. It is clear that a nearly linear relationship exists between the steady-state level of histidase and the growth rate of *A. aerogenes*. It is equally clear, however, that a given reduction in growth rate leads to only half the reduction in histidase level that would result from a tight coupling. Extrapolation of the histidase level to zero growth rate suggests that catabolite repression cannot force the histidase level below 10 units per mg of protein during steady-state growth.

**Unequal functioning of histidase at different growth rates.** The reduction in histidase level is not only less than the reduction in growth rate, it is also slightly less than the reduction in the rate of histidine catabolism. To illustrate the extent of this disparity, the data of Fig. 1 and 2 are replotted in Fig. 3 so that the rate of L-histidine catabolism is shown as a function of the histidase level. Only those experiments in which both parameters were measured on the same cultures are included in Fig. 3. The interrupted line is
TABLE 1. Rate of L-histidine metabolism during biosynthetically restricted growth

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Limiting nutrient</th>
<th>Growth rate (k)</th>
<th>Cell density (C)</th>
<th>L-Histidine inflow ((k_{in}))</th>
<th>L-Histidine outflow ((k_{out}))</th>
<th>L-Histidine used ((k_{in} - k_{out}))</th>
<th>Rate of metabolism ((V_H))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-Arginine</td>
<td>0.226</td>
<td>0.191</td>
<td>1.48</td>
<td>0.545</td>
<td>0.935</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.230</td>
<td>0.198</td>
<td>1.48</td>
<td>0.540</td>
<td>0.940</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.509</td>
<td>0.184</td>
<td>1.48</td>
<td>0.840</td>
<td>0.640</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.655</td>
<td>0.166</td>
<td>1.48</td>
<td>1.00</td>
<td>0.480</td>
<td>1.90</td>
</tr>
<tr>
<td>2</td>
<td>Sulfur</td>
<td>0.208</td>
<td>0.246</td>
<td>3.69</td>
<td>2.23</td>
<td>1.46</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.315</td>
<td>0.230</td>
<td>3.69</td>
<td>2.54</td>
<td>1.15</td>
<td>1.57</td>
</tr>
<tr>
<td>3</td>
<td>Sulfur</td>
<td>0.584</td>
<td>0.162</td>
<td>1.44</td>
<td>0.822</td>
<td>0.618</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.710</td>
<td>0.160</td>
<td>1.44</td>
<td>0.922</td>
<td>0.518</td>
<td>2.30</td>
</tr>
</tbody>
</table>

*a The symbols used in this table are defined in Materials and Methods in reference to equation 5, which has been used to calculate \(V_H\), the rate of L-histidine metabolism. The data presented in this table are a small sampling of the measurements depicted in Fig. 1.

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Fig. 1. Metabolism of L-histidine as a function of growth rate. The specific rate of L-histidine metabolism (expressed as milligrams of L-histidine degraded per hour per milligram, dry weight, of cells) is plotted as a function of the specific growth rate constant, \(k\) (expressed in hr\(^{-1}\)), for many cultures of A. aerogenes LC1 in steady-state growth at 37°C in a chemostat with sulfur limitation (○) or with L-arginine limitation (△), or in batch culture (□). The solid line was drawn by the method of least squares as a line of regression of \(y\) on \(x\).

Fig. 2. Histidase level as a function of growth rate. The specific activity of histidase (expressed as units of enzyme per milligram of total protein) is plotted as a function of the specific growth rate constant, \(k\) (expressed in hr\(^{-1}\)), for many cultures of A. aerogenes LC1 in steady-state growth at 37°C in a chemostat with sulfur limitation (○) or with L-arginine limitation (△), or in batch culture (□). Histidase was assayed in cell-free extracts. The solid line was drawn by the method of least squares as a line of regression of \(y\) on \(x\).
thetic requirements of the cell. (Were histidine catabolism to be reduced proportionately to the growth rate, then the data of Table 1 would have been described by a straight line passing through the origin.) During unrestricted growth, for example, the cells grow at \( k = 0.85 \text{ hr}^{-1} \) by metabolizing L-histidine at a rate of 2.6 mg per hr per mg of cells (Fig. 1); at one-fifth this growth rate (\( k = 0.17 \text{ hr}^{-1} \)) the cells might be expected to grow by metabolizing this substrate at a rate of 0.52 mg per hr per mg of cells. In fact, reference to Fig. 1 shows that their actual rate of L-histidine metabolism at this growth rate is 0.96 mg per hr per mg of cells—nearly double the expected value. A part of this “excess” metabolism might be related to a cellular requirement for maintenance energy, i.e., for the operation of those processes that must occur at rates independent of the growth rate. To discover the contribution of such processes, a culture of LC1 was grown in a chemostat with the carbon and energy source, L-histidine, as the limiting factor. The flow of medium was adjusted to give first a dilution rate (=\( k \)) of 0.144 hr\(^{-1}\), and then one of 0.025 hr\(^{-1}\). Continuous operation at the former rate for 60 hr resulted in a steady-state value of 0.78 for the optical density of the culture; 84 hr at the slower rate led to a steady-state optical density of 0.68 (Table 2). This small difference in culture density during substrate limitation indicates that the amount of histidine metabolized strictly for maintenance is a small fraction of the total histidine metabolism, even at these low growth rates. Therefore, it must be concluded that most of the “excess” rate of L-histidine disappearance observed at low growth rates really does represent an overall imbalance between substrate utilization and biosynthetic demands.

**Fig. 3.** Metabolism of L-histidine as a function of histidase level during growth at different rates. The specific rate of L-histidine metabolism (expressed as milligrams of L-histidine degraded per hour per milligram, dry weight, of cells) is plotted as a function of histidase level (expressed as units of enzyme per milligram of protein) for several cultures of *A. aerogenes* LC1 in steady-state growth in a chemostat with sulfur limitation (\( \Delta \)) or with L-arginine limitation (\( \circ \)), or in batch culture (\( \square \)). The data include all cultures shown in Fig. 1 and 2 for which both histidase level and substrate metabolism were measured. The solid line was drawn by the method of least squares as a line of regression of \( y \) on \( x \). The interrupted line is drawn from the origin to the coordinates characteristic of the batch culture.

**Table 2.** Cell density and histidase level at low dilution rates in an L-histidine restricted chemostat

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Dilution rate (d)</th>
<th>Optical density (430 nm)</th>
<th>Histidase level (units per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.144</td>
<td>0.80</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>0.025</td>
<td>0.72</td>
<td>32.9</td>
</tr>
<tr>
<td>0</td>
<td>0.025</td>
<td>0.78</td>
<td>34.1</td>
</tr>
<tr>
<td>0</td>
<td>0.025</td>
<td>0.80</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>0.025</td>
<td>0.68</td>
<td>31.7</td>
</tr>
</tbody>
</table>

* A culture of *A. aerogenes* LC1 was grown in a chemostat in a medium containing excess sulfur and L-arginine (40 \( \mu \text{g}/\text{ml} \)), but limiting L-histidine (500 \( \mu \text{g}/\text{ml} \)).

* The histidase level was determined in extracts of cells from the effluent culture collected over several hours of growth and held at 0°C. As a control, a portion of the culture growing at a \( k \) of 0.025 was inoculated into normal batch medium and grown for several generations, after which their histidase level was found to be 23.2 units per mg of protein.

* An arbitrary time after steady state had been reached at a dilution rate of 0.144 hr\(^{-1}\).

* The time at which the dilution rate was changed from 0.144 hr\(^{-1}\) to 0.025 hr\(^{-1}\).
in a sulfate-limited chemostat at growth rates of 0.2 and 0.7 hr⁻¹. The flow of carbon from substrate into cells, medium, and volatile material was measured by procedures described in Materials and Methods. The results are displayed in Tables 3 and 4.

In rapid growth (k = 0.71 hr⁻¹), the cells consumed 6.57 μmoles of L-histidine carbon per hr per mg of cells. Of this, 44% was assimilated as cell carbon, and 56% was excreted as formamide (17%, calculated), CO₂ and other volatile material (33%), and unknown nonvolatile compounds (6%). During slow growth (k = 0.21 hr⁻¹), the same mass of cells consumed L-histidine carbon at the rate of 3.71 μmoles per hr. They assimilated only 20% of this carbon, the remaining 80% being excreted as formamide (17%, calculated), CO₂ and other volatile material (42%), and unknown nonvolatile compounds (21%). Overall, these results are consistent with the previous measurements; also, they provide an indication that metabolic by-products may be excreted in different proportions during fast and slow growth.

It was of interest to learn whether these differences would be preserved after the cells had been prepared by growth at two different rates and then transferred to identical “resting” conditions. Two kinds of experiments were performed. In the first, the resting-cell suspensions were presented with uniformly labeled ¹⁴C-L-histidine, and the fate of the L-histidine carbon was examined as in the chemostat experiments. The results are shown in Tables 3 and 4. There was, of course, very little assimilation of carbon by either the fast- or the slow-grown cells. The suspensions of cells preserved, more or less unchanged, their relative rates of histidine degradation during growth (6.1

<table>
<thead>
<tr>
<th>Table 3. Catabolism of ¹⁴C-L-histidine by resting and growing cultures of cells previously grown at a fast or slow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate to prepare cells, k (hr⁻¹)</td>
</tr>
<tr>
<td>--------------------------------------</td>
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<tr>
<td>Growing-cell experiment</td>
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<tr>
<td>0.210</td>
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<td>0.714</td>
</tr>
<tr>
<td>Resting-cell experiment</td>
</tr>
<tr>
<td>0.190</td>
</tr>
<tr>
<td>0.850</td>
</tr>
</tbody>
</table>

* For the growth experiment, the figures represent the chemical concentration (measured colorimetrically) and the radioactivity (uniformly labeled ¹⁴C-L-histidine) of the medium flowing into the chemostat. For the resting, or nongrowth experiment, they represent the values at the start of incubation of the cell suspensions.

† For the growth experiment, the figures describe the material flowing out of the chemostat; for the nongrowth experiment, they describe the contents of the incubation mixture at the end of the experiment—8.0 hr in the case of the slow cells, and 2.5 hr in the case of the fast cells.

‡ The dry weight is calculated from the optical density of the culture; the radioactivity was measured by filtering the cells from the medium.

The radioactivity of the filtered medium was assumed to consist of (i) unmetabolized L-histidine, (ii) formamide, and (iii) other nonvolatile by-products of L-histidine metabolism. The radioactivity associated with L-histidine was calculated from the L-histidine remaining (measured colorimetrically) and its specific activity; that associated with formamide was calculated as one-sixth of the radioactivity of the L-histidine consumed (12); the radioactivity of other by-products in the medium was then calculated by subtraction.

In the growing-cell experiment, samples of the gas that had been used to aerate the growth chamber were bubbled through KOH solutions for 30- and 60-min periods; the results shown here are the average of all such samples and are expressed as counts per minute of CO₂ produced by 1 ml of culture in 1 hour. In the resting-cell experiment, all of the CO₂ produced was trapped in the KOH in the center well; the results shown are expressed as counts per minute of CO₂ produced by 1 ml of the culture in 8 hr by the slow cells and in 2.75 hr by the fast cells. The “nonrecovered” counts represent radioactivity which is presumed to be associated with volatile material either not trapped by KOH or not precipitated by BaCl₂.
The second type of experiment consisted simply of a classical analysis of respiration and substrate utilization in a Warburg apparatus. Two suspensions of LCl cells were prepared, one by growth at \( k = 0.19 \) hr\(^{-1}\) in unrestricted histidine medium and the other by growth at \( k = 0.19 \) hr\(^{-1}\) in histidine medium in a sulfur-restricted chemostat.

The histidine content of the cells and their rates of histidine utilization, of \( \text{O}_2 \) uptake, and of \( \text{CO}_2 \) production were then measured as described in Table 5 and Materials and Methods. The results, in general, confirm the isotope experiment, although the fraction of carbon converted to \( \text{CO}_2 \) was higher in both slow (35%) and fast (23%) cells than was seen in the isotope experiment. The reason for this is unknown, but may possibly be poorer aeration in the large vessel used in the isotope experiment, or poorer recovery of \( \text{CO}_2 \) produced. Nevertheless, the results do demonstrate that slow-grown cells metabolize histidine more slowly and may possibly oxidize it more completely than do fast-grown cells.

This hint that L-histidine may be metabolized in a different way during biosynthetically restricted growth than during batch growth suggests an explanation for the failure of catabolite repression to effect a strict coordination between histidine synthesis and growth rate. A steady-state condition may be reached in which potentially repressing catabolites are converted into nonrepressing by-products, such as \( \text{CO}_2 \).

### Table 4. Comparison of distribution of carbon during metabolism of \(^{14}\text{C}-\text{L-histidine} \) by cells grown at fast or slow rates

| Type of cells | Incubation condition | Percentage distribution of metabolized carbon |  |
|--------------|----------------------|---------------------------------------------|  |
|              |                      | Cell material | Formamide* | Unknown | CO₂ | Unknown* |
| Slow         | Slow growth          | 20            | 17          | 21      | 20  | 22       |
|              | Nongrowth            | 6             | 17          | 35      | 22  | 20       |
| Fast         | Fast growth          | 44            | 17          | 6       | 30  | 3        |
|              | Nongrowth            | 6             | 17          | 52      | 15  | 10       |

* The percentage distributions of metabolized L-histidine carbon atoms shown in this table were calculated from the data of Table 3.

* Calculated as one-sixth of the metabolized carbons.

* These figures represent unrecovered radioactivity presumed lost in the gas phase.

\( \mu \text{moles of carbon per hr for the fast cells and } 2.0 \mu \text{moles for the slow cells} \). The cells that had prepared by slow growth converted relatively more of the histidine carbon to \( \text{CO}_2 \) and other volatile compounds than did the cells prepared by rapid growth.

### Table 5. Manometric measurements of L-histidine metabolism in suspensions of cells grown at fast and slow rates

<table>
<thead>
<tr>
<th>Preparation* of cells</th>
<th>Histidase* level (units per mg of protein)</th>
<th>Rates of metabolism* (( \mu \text{moles per hr per mg of cells} ))</th>
<th>( \frac{V_{\text{CO}<em>2}}{V</em>{\text{H}}^{\text{a}}} \frac{a}{a} )</th>
<th>CO₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow grown (( k = 0.20 ))</td>
<td>13.1</td>
<td>4.33</td>
<td>10.2</td>
<td>42</td>
</tr>
<tr>
<td>Fast grown (( k = 0.85 ))</td>
<td>22.3</td>
<td>9.09</td>
<td>13.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* A chemostat culture of \( A. \text{aerogenes} \) LCl was prepared in steady-state growth at \( k = 0.19 \) hr\(^{-1}\) with sulfur limitation. One portion was then grown in normal media at a \( k = 0.85 \) for several generations. The two cultures were then harvested; the cells were collected and washed by centrifugation, and were then suspended in basal salts medium (lacking both L-arginine and sulfur). Portions (3 ml) of the suspensions were placed in Warburg vessels, a known quantity of L-histidine was added, and manometric measurement was begun. The suspension of slow cells contained 0.109 mg of cells per ml; the suspension of fast, 0.106 mg of cells per ml.

* Portions of each cell suspension were used to prepare extracts for the measurement of histidase.

* At the conclusion of the manometric measurements, the quantity of L-histidine remaining in each flask was measured colorimetrically, and the consumption of substrate was used to calculate \( V_{\text{H}} \), the specific rate of L-histidine metabolism. From the manometric data, \( V_{\text{CO}_2} \) and \( V_{\text{O}_2} \), the specific rates of \( \text{CO}_2 \) evolution and of \( \text{O}_2 \) uptake, respectively, were calculated.

* The ratio, \( V_{\text{CO}_2}/V_{\text{H}}^{a} \), is a measure of the number of micromoles of \( \text{CO}_2 \) produced per micromole of L-histidine metabolized.

* The assumption was made that a molecule of L-histidine has five carbon atoms that can possibly be converted into \( \text{CO}_2 \) (the sixth appearing as formamide); then, from the ratio \( V_{\text{CO}_2}/V_{\text{H}}^{a} \), the \( \text{CO}_2 \) formed was expressed as a percentage of that which could be formed by complete metabolic combustion.
Histidase synthesis following a decrease in growth rate. If fast and slow cells differ in their enzymatic capacity to handle excess catabolites, then cells that have been growing rapidly should exhibit greater catabolite repression immediately after a shift down in growth rate than later, after the alternate pathway has been developed. To examine this possibility, cells of strain LCI were grown in a chemostat in L-histidine minimal medium with L-arginine as the limiting factor. Initially the culture was grown at $k = 0.66 \text{ hr}^{-1}$, which is 80% of the maximum possible growth rate. After steady-state conditions were achieved, the dilution rate was abruptly changed from 0.66 hr$^{-1}$ to 0.13 hr$^{-1}$, and samples of the effluent culture were collected at 30-min intervals. The optical density, and the total protein content of these samples were measured, and then the cells were assayed for their histidase content (Fig. 4). The very slight increase in optical density and total protein per milliliter of culture is a normal response of chemostat cultures to decreased flow rate. From the upper panel of the figure, it can be seen that the histidase content of the cells emerging from the chemostat began to drop immediately after growth was slowed. The physical nature of a chemostat is such that the time course of transient responses is not easily followed; samples cannot be removed at discrete times from the growth vessel, but rather must be collected by accumulation of effluent culture over a finite time period (in the present case, 15 min). Nevertheless, it can be judged from Fig. 4 that the histidase level approaches its new steady-state value at a rate suggesting that no new enzyme is made for 5 hr.

For a full generation at slow growth rates, therefore, biosynthetic restriction is capable of effecting a total repression of histidase formation. Closely similar results were obtained when sulfate rather than L-arginine restriction was used to limit growth.

Growth and histidase synthesis following release of a biosynthetic restriction. It appears that the full extent of catabolite repression on histidase formation is not maintained during slow growth, presumably because there is both a reduction in the rate of L-histidine degradation per unit of histidase activity and a quantitative or qualitative change in the by-products produced from L-histidine. It was of interest, therefore, to learn how the cells would respond to a sudden release of biosynthetic restriction after steady-state growth at a slow rate had been established. A culture of strain LCI in steady-state growth at $k = 0.13 \text{ hr}^{-1}$ was prepared in a chemostat on limiting L-arginine. To begin the experiment, the dilution rate was abruptly increased fivefold from 0.13 to 0.66 hr$^{-1}$. The optical density and total protein content of the effluent culture were monitored, and cell samples accumulated every 10 min were assayed for histidase. Many such experiments were performed, some with L-arginine limiting and some with sulfate limiting the growth. Typical results are shown in Fig. 5. The upper panel depicts the histidase level in the cells; it is clear that the sudden removal of the L-arginine restriction led to a rapid derepression of this enzyme. From the information given in Fig. 5, it can be calculated (see Materials and Methods) that the differential rate of histidase formation during the first 30 min after the shift is 33 units/mg of protein—a value in close agreement with that observed during substrate-restricted growth (Table 2).

But it is the growth response of the cells that provides the most interesting result of this experiment. From the lower panel of Fig. 5, it can be seen that the cell mass (as measured both by optical density and by total protein) per milliliter of culture began to decrease as soon as the dilution rate was stepped-up. This decrease indicates that the cells were, for almost 2 hr, unable to grow as fast as the dilution rate (0.66 hr$^{-1}$). Their actual rate of growth, calculated as described in Materials and Methods, was 0.40 hr$^{-1}$ during the first 30 min after the shift. Since this
rate is established almost instantaneously after the shift, i.e., before a significant increase in histidase level has occurred, the cells must quickly have been able to make three times more efficient use of the histidase they already possessed at the slow growth rate.

It was tempting, therefore, to calculate how fast the cells could have grown immediately after removal of the biosynthetic restriction if (i) their histidase complement were to function as actively as it does during rapid (unrestricted) growth and also (ii) they were to begin converting L-histidine to cell material with the efficiency characteristic of rapid growth. In batch culture, these cells contain 24 units of histidase per mg of protein, they metabolize L-histidine at a rate of 2.7 mg per hr per mg of cells, and they grow at a rate of 0.85 hr\(^{-1}\) (see Fig. 1 and 3). In the shift-up experiment just described, the cells contained 14 units of histidase at the time of the shift up; they should, therefore, have been able to metabolize L-histidine at a rate of 1.58 mg per hr per mg of cells and to grow at a rate of 0.49 hr\(^{-1}\). The agreement between this growth rate and that actually achieved (0.40 hr\(^{-1}\)) strongly suggests that our assumption of full functioning of histidase and conversion of L-histidine to protoplasm at batch rate efficiency is very nearly correct. Four additional shift-up experiments were performed. In all of them, the cells were able to triple their growth rate instantaneously, and to achieve a value close to but never exceeding the theoretical maximum based on their histidase content (results not shown). Finally, experiments were performed in which the dilution rate of the chemostat was stepped up about 2.5-fold—a value that should be within the capacity of the cells' growth response. As shown in Fig. 6, such shifts resulted in little or no perceptible decrease in cell density, confirming the fact that growth can be accelerated from \(k = 0.15\) to \(k = 0.42\) by nearly instantaneous metabolic adjustments.

**DISCUSSION**

Restriction of growth in *Aerobacter* clearly does affect substrate catabolism. Let us trace what happens to the metabolism of L-histidine as we take a culture of *A. aerogenes* in steady-state fast growth and decrease its growth rate by means of some biosynthetic restriction. First, upon imposing the restriction there will be no more than a 10% reduction in the specific rate of L-histidine breakdown (deduced by comparing \(V_b\) values from Table 1 with Table 5); in other words, catabolism will greatly exceed anabolism during this transient phase. The formation of histidase and presumably other catabolite-sensitive enzymes will be totally arrested, and continued growth at the new slow rate will dilute out the cells' complement of histidase (cf. Fig. 4). This gradual reduction in the level of histidase [and probably at least that of the next enzyme in the pathway as well (9)] gradually reduces the specific rate of L-histidine metabolism.

At the same time, two other processes seem also to come into play. One of these contributes further to the reduction in L-histidine metabolism by somehow slightly restricting the operation of the histidase pathway (cf. Fig. 3). Since histidase requires no coenzymes and catalyzes a virtually irreversible reaction (9), it is difficult to envisage how the rate of L-histidine metabolism could be
inhibited except by mechanisms that affect the activity of this enzyme directly (e.g., by end-product inhibition) or indirectly (e.g., by restricted entry of L-histidine into the cells). We have not examined the histidase of this organism for possible allosteric interactions, but the equivalent enzyme in \textit{Pseudomonas aeruginosa} has a sigmoid substrate saturation curve, and is partially inactive in vivo under certain growth conditions (6). In \textit{P. putida}, there is evidence that succinate inhibits urocanase, the second enzyme in the pathway, and that urocanate inhibits histidase, leading to a sequential negative feedback inhibition (3).

The third process that seems to occur as the cells adjust to the slower rate of growth is an alteration in the capacity of the cell to dispose of excess catabolites. After about a generation of growth with complete catabolite repression of histidase, synthesis of this enzyme resumes at a relaxed rate, i.e., at a rate that leads to no more than a 50\% coupling of histidase formation to growth rate. We interpret this relaxation as being the result of an expanded capacity of the cell to convert repressing catabolites into inert by-products (e.g., ethyl alcohol, CO\textsubscript{2}, lactate, etc.), thereby maintaining the histidase level at a higher value than actually required for growth at the new rate. Indirectly, this relaxation also is responsible for the decreased growth yield. We have no solid information about the nature of this presumed adjustment in catabolite metabolism, nor of the biochemical entities involved. It is unnecessary that such adjustment cause a qualitative change in the nature of the by-products excreted by the cell, since the adjustment might entail simply a coordinate expansion of pathways already used at high growth rate. Nevertheless, evidence for a qualitative change would be welcome, because it would make more convincing the argument that the internal level of catabolites is thereby kept lower during steady-state slow growth than during the period of adjustment just after the shift. Our finding that cells adjusted to slow growth seem to excrete a larger portion of their carbon as volatile material provides a preliminary indication that such a difference may exist, but more precise analyses are necessary before this can be interpreted.

Finally, a new steady state is reached in which, compared with rapid growth, (i) the cells consume less L-histidine per hr per mg of cells, (ii) they produce less histidase per mg of protein, (iii) the histidase they possess functions at a slightly lower overall rate in vivo, and (iv) a different pattern of by-products may possibly be produced from the substrate.

To complete our description of how growth affects catabolism, consider next what happens when the restriction on biosynthesis is removed and the cells are permitted to return to their batch growth rate. The cells almost instantaneously increase their rate of histidase formation, as would be predicted by the release of catabolite repression, but long before the level of enzyme has been significantly elevated the cells achieve a near-tripling in growth rate. This ability to grow faster with the existing level of histidase must mean that both the restriction on L-histidine metabolism and the diversion of carbon to excreted by-products can be quickly reversed.

This behavior seems complex, but upon reflection one can see some physiological sense in it. Consider how catabolism should be controlled. There are two requirements that must be met: (i) cells should be spared from synthesizing large quantities of unnecessary enzymes, but at the same time (ii) each of the three major products of catabolism (intermediary metabolites, energy in the form of ATP, and reducing power in the form of reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate) must be supplied at an appropriate rate to the biosynthetic machinery of the cell.

The former requirement can, of course, be met by catabolite repression, which prevents the induction of unneeded enzyme species and which, as we have seen here, must be potentially capable of maintaining the minimal level of catabolic enzyme consistent with a given rate of cellular biosynthesis, since the rate of histidase formation is reduced to near zero after a shift down.

The latter requirement poses the usual problem of how to control branching pathways that have multiple end products. The problem can be particularly acute in this case, for the relative demand for carbon compounds and ATP must vary enormously under different growth conditions. In a medium rich in the end products of biosynthesis (e.g., amino acids, nucleotides, and vitamins) intermediary metabolites will not be drained off into biosynthetic pathways at an appreciable rate. And yet the energy demands for growth in such a medium are considerable because of the large energy requirement for macromolecular synthesis. Therefore, the potential accumulation of repressing catabolites must be prevented, or a disastrous curtailment of energy production will result. One way to solve this problem would be to bypass catabolite repression by converting all products of catabolism into such (presumably nonrepressing) by-products as CO\textsubscript{2}, lactate, formate, ethyl alcohol, and acetate. Excretion of these compounds would then permit the level of catabolic enzymes, and therefore the rate of catabolism, to remain high enough to satisfy the energy demands of the cell. Therefore, the second requirement for any general control on catabolism...
could be met by the existence of pathways that would be induced by high levels of intermediary catabolites and that would dispose of these catabolites. The full effect of catabolite repression would then be evident only during the transient period required to form or expand the new pathway.

The results of Rosenberger and Elsdon (14) are consistent with this picture. Their results were obtained with a rich, anaerobic medium in which glucose was almost quantitatively converted to lactic acid at each growth rate. In a medium which supplies all or most of the monomeric subunits of protoplasm, no catabolites are used for biosynthesis, irrespective of the growth rate. Therefore, as Rosenberger and Elsdon found, the rate of glucose degradation may well be independent of growth rate in this situation, for whatever effect catabolite repression has in this organism should already have been present during rapid growth. Such a result implies, of course, that ADP is regenerated by direct or indirect adenosine triphosphatase systems which themselves must be under rather stringent (and interesting) control.

When the growth of a cell is forced to a lower rate by a biosynthetic restriction, the regulatory signal seen by the cell is, at least in part, the same as if a supplement rich in building blocks had been added to the culture—a transiently high concentration of catabolites. Our results suggest that the response of the cell may be similar in these two conditions: first, a period of growth during which these catabolites both repress substrate-degrading enzymes and induce catabolite-degrading enzymes, and then steady-state growth in which a balance is achieved, the substrate-degrading enzymes being partially repressed.

Mention was made in the introduction of several instances (1, 4, 12, 15) in which continued rapid glucose catabolism has been observed when nitrate or molecular nitrogen was substituted for ammonia as the nitrogen source, even though the growth rate was reduced. In at least one of these cases, that of *A. aerogenes* growing with nitrate, we have been able to demonstrate (11) that the increased glucose catabolism is still sensitive to changes in the growth rate. In the other cases, less can be said with certainty, but we would suggest that catabolite repression might operate transiently in these organisms, as it does in *Aerobacter*, and then be bypassed even more completely by expansion of a metabolic route to dispose of excess metabolites.

In summary, this study of l-histidine catabolism in *A. aerogenes* has confirmed the premise that cells make a regulatory response to an imbalance between catabolism and biosynthesis. In this organism, the response consists chiefly of catabolite repression, which is severe at first and then becomes relaxed, possibly by the expansion of pathways that convert catabolite repressors into inert by-products. In addition, at very low growth rates the l-histidine-degrading enzymes appear to function somewhat less rapidly than at high growth rates.

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**LITERATURE CITED**