Growth characteristics of mutant strains of Escherichia coli

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GROWTH CHARACTERISTICS OF MUTANT STRAINS
OF ESCHERICHIA COLI

by

Isaac Francis Baarda

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1962
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INTRODUCTION

The multiplication of all organisms, whether plant or animal, single- or multicellular, has as a common feature the process of replication of a genetic and protoplasmic organization to yield two more or less identical structures. At first glance the process of duplicating a highly specialized, multicelled organism would appear to be vastly more complex than that of duplicating a bacterium. However, with recent advances in cytological and biochemical techniques and many reports of the complexity of particular parts of the bacterial cell (Salton, 1961; Strominger, 1960), its metabolic processes (Stanier, 1961; Roberts et al., 1955; Wallen et al., 1959; Petty, 1961) and its genetic constitution (Lederberg, 1957), the concept of "simple" bacterial growth and replication may prove to be only a myth.

The growth of bacteria in batch culture, containing only a limited amount of nutrient material, follows a generally predictable pattern. This order of events consists of a lag phase, during which the cells increase in protoplasmic mass and size but do not increase in numbers, an exponential growth phase, during which the cells are dividing at their maximal rate, and a stationary phase, which is produced by the imposition of some limiting
environmental factor upon exponential growth. Following this there is a death phase during which the total number of viable cells gradually declines.

The transition periods between lag phase and exponential growth, and between exponential growth and stationary phase, are of great interest to investigators concerned with growth and morphogenesis. The cytological (Chance, 1959), physiological (Lichstein, 1959) and mathematical (Ecker, 1961a) aspects of the lag phase and its transition to exponential growth have been studied in detail.

The transition between exponential and stationary phases might be thought of as a reversal of the transition between lag phase and exponential growth. It has been noted by several authors that there are definite differences, cytological and biochemical, between cells in the exponential and the stationary phases in batch cultures. Lockhart and Powelson (1954) found changes in cell size, nucleic acid content, ratio of ribonucleic acid to deoxyribonucleic acid, sensitivity to chemical agents and respiratory activity of the cells taking place during this transition phase. Kopper (1952) demonstrated variations in reducing activity, temperature sensitivity, pH optimum for reducing activity, and sensitivity to chemical agents between exponential and stationary phase cells.
Cohen (1951) indicated that resting cells of *Escherichia coli* dissimilated glucose by the Embden-Meyerhof pathway while rapidly growing cells used chiefly the hexosemonophosphate shunt. In glucose-limited cultures, Allen and Powelson (1957) demonstrated that exponentially growing cells of *E. coli* metabolized 86% of the glucose used by a C-1 preferential pathway, considered to be the hexosemonophosphate shunt, while in early stationary phase only 29% of the glucose used was metabolized via this pathway. In nitrogen-limited cultures exponentially growing cells oxidized 80% of the glucose used by the C-1 preferential pathway while during stationary phase only 53% of the glucose taken up was oxidized in this manner. Similar results have been found with other microorganisms (Beevers and Gibbs, 1954; Heath and Koffler, 1956). In reviewing these phenomena, Lockhart (1959) suggested that such changes may be analogous to cellular differentiation in higher organisms.

Such changes obviously conform to the definition of biochemical differentiation as ..."a change in the enzymic pattern of the cell ....." (Mandelstam, 1960). The work reported in this thesis is an attempt to delineate the differences between a group of closely related bacterial strains in their exponential, transitional and stationary phases in a closely controlled growth environment.
Detection of some of the interrelationships between the mechanisms of growth control and biochemical differentiation at the cellular level was the final goal.
MATERIALS AND METHODS

General

The organisms used in this work were *Escherichia coli*, strain K12, and several substrains derived from this organism. Stock cultures were maintained at 50°C on slants of minimal agar (Davis and Mingioli, 1950) and transferred semiannually.

Cultivation of these organisms for experimental purposes was in a synthetic medium containing: $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.7%; $\text{KH}_2\text{PO}_4$, 0.3%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01%; deionized water; and varying amounts of $(\text{KH}_4)_2\text{SO}_4$ (Baker, Reagent grade, granular) and glucose (Merck, Reagent grade) to attain the growth-limiting or -nonlimiting concentrations desired. The glucose was made up separately in stock solution, autoclaved, and added aseptically to the medium just before inoculation. pH of the medium after autoclaving was 6.9-7.1.

Inocula were 10 ml volumes of A broth (minimal broth with 0.05% sodium citrate added) inoculated from a stock slant and incubated at 37°C for 23 to 25 hrs without aeration. After incubation the cells were separated by centrifugation in an angle centrifuge at 1500 x g for 20 min and resuspended in 5 to 10 ml of pH 7.0 Sorenson's
phosphate buffer. The correct volume of this suspension was added to the culture vessel to give an inoculum of \(1.7 \times 10^7\) cells/ml (\(\log_2 \text{cells/ml} = \log_2 G_0\)).

The organisms were cultivated in 10 ml volumes of medium in 18 x 150 mm culture tubes aerated by single orifice gas delivery tubes or in 500 ml volumes of medium in straight walled, one L Pyrex bottles aerated through Pyrex fritted glass gas dispersion tubes of coarse porosity. Sterilization of the air was accomplished by passage through a type WH Microweb filter (Millipore Filter Corp., Watertown, Mass.) placed over the inlet of the delivery tube or by filtration through a sterile glass wool filter. The air flow metering devices and culture vessels have been previously described (Lockhart and Ecker, 1959; Ecker and Lockhart, 1961a). Oxygen absorption rates (OAR) were determined by a modification of the sulfite oxidation technique as described by Ecker and Lockhart (1959b). Aeration rates were always maintained at an OAR of not less than 40 m moles \(O_2/L/hr\), a rate which will support a population of \(1 \times 10^{11}\) cells of \(E. \text{coli} \text{K12}\) per ml of synthetic medium (Ecker and Lockhart, 1961b). The maximum population achieved in any experiments reported here was never more than 5% of this value.

Samples taken for various determinations were centrifuged at 1500 x g for 20 min and the cells and supernatant
were separated immediately. If determinations were to be made within 48 hrs the samples were held at 5° C; for longer storage periods the samples were stored in a deep freeze at -15° C. When aliquots for analysis were removed the samples were thawed rapidly and refrozen as soon as possible. It has been demonstrated that such storage procedures do not affect the analytical determinations (Ecker and Lockhart, 1961b).

Isolation Methods and Characteristics of Mutant Strains

The mutant strains studied were isolated in this laboratory using two general methods. The first was based on the process of orthoselection (McDonald, 1955) by serial cultivation under various limiting conditions and the second was based on the agar gradient plate method and was used to select drug resistant mutants.

The parent strain and all the mutant strains listed in Table 1 were facultatively anaerobic, motile, nonsporeforming, gram negative rods which ranged from 0.5 to 2.0 microns in length. The colonies were white to yellowish, moist, glistening, spreading growths which had a definite fecal odor when grown on complete medium. The various differential tests showed that indole was produced, the methyl red test was positive, the Voges-Proskauer test was negative,
Table 1. Isolation conditions and characteristics of selected mutant strains of *Escherichia coli*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Basis of isolation</th>
<th>Conditions of isolation</th>
<th>Number of transfers or level of drug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12</td>
<td>wild</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>K12/G1</td>
<td>orthoselection</td>
<td>nutrient broth</td>
<td>75 transfers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unaerated</td>
<td></td>
</tr>
<tr>
<td>K12/G2</td>
<td>orthoselection</td>
<td>minimal agar slants</td>
<td>62 transfers</td>
</tr>
<tr>
<td>K12/G3</td>
<td>orthoselection</td>
<td>X broth (^a)</td>
<td>31 transfers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unaerated</td>
<td></td>
</tr>
<tr>
<td>K12/G4</td>
<td>orthoselection</td>
<td>X broth OAR = 24.2 (^b)</td>
<td>33 transfers</td>
</tr>
<tr>
<td>K12/G5</td>
<td>orthoselection</td>
<td>X broth OAR = 16.3</td>
<td>35 transfers</td>
</tr>
<tr>
<td>K12/G6</td>
<td>orthoselection</td>
<td>X broth OAR = 41.4</td>
<td>34 transfers</td>
</tr>
<tr>
<td>K12/G7</td>
<td>orthoselection</td>
<td>Y broth (^c)</td>
<td>35 transfers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unaerated</td>
<td></td>
</tr>
<tr>
<td>K12/G8</td>
<td>orthoselection</td>
<td>Y broth OAR = 24.7</td>
<td>35 transfers</td>
</tr>
<tr>
<td>K12/G9</td>
<td>orthoselection</td>
<td>Y broth OAR = 17.3</td>
<td>35 transfers</td>
</tr>
</tbody>
</table>

\(^a\)X broth contains \(1 \times 10^3 \text{ } \mu g/ml \text{ (NH}_4\text{)}_2\text{SO}_4\) and \(3 \times 10^3 \text{ } \mu g/ml \text{ glucose.}\)

\(^b\)OAR is oxygen absorption rate and is expressed in m moles \(O_2/L/hr\).

\(^c\)Y broth contains \(5 \times 10^2 \text{ } \mu g/ml \text{ (NH}_4\text{)}_2\text{SO}_4\) and \(5 \times 10^3 \text{ } \mu g/ml \text{ glucose.}\)
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Basis of isolation</th>
<th>Conditions of isolation</th>
<th>Number of transfers or level of drug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12/G10</td>
<td>orthoselection</td>
<td>Y broth, OAR = 39.5</td>
<td>34 transfers</td>
</tr>
<tr>
<td>K12/A4</td>
<td>NaAsO$_2$ resistance</td>
<td>nutrient agar gradient plate, 0.008 M NaAsO$_2$</td>
<td></td>
</tr>
<tr>
<td>K12/N5</td>
<td>$\beta$-naphthylamine resistance</td>
<td>nutrient agar gradient plate, 0.0022 M $\beta$-naphthylamine HCl</td>
<td></td>
</tr>
<tr>
<td>K12/TU2</td>
<td>thiourea resistance</td>
<td>serial transfer in nutrient broth plus 1.5% thiourea</td>
<td></td>
</tr>
</tbody>
</table>

$^d$NaAsO$_2$ is Baker and Adamson Reagent grade, Powder.

$^e$$\beta$-naphthylamine HCl from Eastman Kodak Company.

$^f$Thiourea is from Eastman Organic Chemicals, Lot No. 12.

acid and gas were produced from glucose, maltose and lactose, citrate could not be used as the sole carbon source and nitrite was not produced from nitrate. Some strains would not grow in the presence of 6.5% NaCl while others grew only after 48 to 96 hrs incubation. The long incubation periods may be indicative of the selection of resistant mutants.

The conditions for isolation of the various mutants:
and the number of transfers (in the case of the ortho-selected mutants) or the level of drug to which the organism was resistant, are given in Table 1. Transfers were made at 12 to 24 hr intervals. The resistance of the parent strain to sodium arsenite was 0.004 M, to \( \beta \)-naphthylamine 0.002 M and to thiourea 1.36%. The strains studied in greatest detail were the wild type K12, K12/A4, K12/G3, K12/G6, K12/G8 and K12/G9.

Analytical Procedures

Total counts were made using a membrane filter technique (Ecker and Lockhart, 1959a). This method consisted of filtering out a known volume of diluted and chemically fixed cells on a defined area of a type HA millipore filter (Millipore Filter Corp., Watertown, Mass.), staining these cells with 0.1% aqueous acid fuchsin, drying the filter and microscopically counting the cells in a prescribed area. Multiplication of this count by the proper dilution factors gave the number of cells/ml in the original culture sample.

Cell size distribution data were obtained by measuring the length of 100 stained cells in a wet mount using a Whipple ocular micrometer. The procedure consisted of placing six small drops of dilute safranin stain on a microscope slide and allowing them to dry. After the drops were dry a loopful of the culture to be examined was placed on a
dye spot and covered with an 11 by 11 mm square of Corning cover glass, No. 2. The edges of the cover slip were sealed with melted parawax to prevent evaporation and the cells examined and measured using the oil immersion objective. Only cells which appeared to be lying in a plane at right angles to the line of sight were measured and the length was expressed in arbitrary units based upon the distance between the lines of the smallest grid of the Whipple ocular micrometer. The results were expressed either as numbers of cells plotted against length, or by calculating the average cell length.

Optical density and nephelometric measurements were made using a Coleman Model 14 Universal spectrophotometer (Coleman Instruments Inc., Maywood, Ill.) which had been modified by addition of a 2 by 10 mm slit between the terminal collimating system and the sample chamber. This modification reduced the spectral width of the beam and diminished the stray light. For nephelometry a Coleman nephelometer adapter and a 20 x 40 mm cuvette were used and for optical density measurements the standard Coleman 19 mm round cuvettes, types A and B, were used.

Total protein was determined by two methods. The first (Phenol method or Phenol protein) was based upon the method of Lowry et al. (1951) as modified by Ecker and Lockhart (1961c). To a sample containing $10^7$ to $10^8$ cells, centrifuged
and resuspended in Sorenson's phosphate buffer in 0.5 ml total volume, or to 0.5 ml culture filtrate, 0.3 ml of 4.0 M NaOH was added. This was heated in a boiling water bath for 4 min, cooled rapidly, and 0.2 ml of 4.0 M HCl added. This sample was then treated after the manner of Lowry et al. (1951). A standard of crystalline bovine serum albumin (Armour Labs, Chicago, Ill.) was included with each test series and the results expressed as µg serum albumin per ml of original culture. Figure 1 shows the resulting curve when various concentrations of cells of E. coli K12 grown in minimal media without aeration were analyzed for total protein by this method.

The second method of total protein determination was based upon the biuret reaction (Layne, 1957). A sample of culture fluid containing 2 x 10⁹ to 8 x 10⁹ cells was taken, and the cells centrifuged as described previously. After removal of the supernatant fluid a 2 ml volume of 1.0 M CO₂ free NaOH was added and the cells resuspended and heated for 4 min in a boiling water bath. The samples were cooled and 4 ml of Biuret reagent added and mixed. The color was allowed to develop for at least 15 min at room temperature and the optical density taken at 550 μ against a 1 M NaOH blank treated as a sample. A curve of various concentrations of bovine serum albumin was included with each set of samples. Figure 1 shows three standard
Figure 1. Standard curves for phenol and biuret protein determinations

A - Phenol protein:

Solid circles - phenol protein determinations of dilutions of cells of *Escherichia coli* K12

B - Biuret protein:

Open circles - biuret protein determination of *α*-lactoglobulin

Open squares - biuret protein determination of dilutions of cells of *Escherichia coli* K12

Open triangles - biuret protein determination of dilutions of cells of *Escherichia coli* K12
curves of a series of samples containing $\beta$-lactoglobulin, bovine serum albumin or cells of E. coli K12. As can be seen, the greatest deviation from a straight line is not more than 5% for the cell dilutions or for the pure protein samples.

Total extractable nucleic acid in the cells or total nucleic acid in the culture filtrate were determined by modifying the technique of Ogur and Rosen (1950) to apply to the culture systems used here. A 1 ml sample of a cell suspension containing about 100 $\mu$g protein per ml, or a 1 ml sample of culture filtrate, was mixed with 4 ml of 0.5 N perchloric acid and heated in a water bath at 70° C for 20 min. The cells or sediment were separated, if necessary, by centrifugation and the optical density values of the supernatant at 260 mp and 280 mp were obtained using a Beckman Model DB spectrophotometer and matched 1 x 1 cm silica cuvettes. The blank consisted of 0.5 N perchloric acid. The concentration of nucleic acid was found using a nomograph based upon the data given by Warburg and Christian (1942). No attempt was made to distinguish between ribonucleic acid and deoxyribonucleic acid concentrations. Extractable or soluble protein was estimated by the same procedure.

The pH values of culture filtrates were determined using a Beckman Model G pH meter calibrated against Beckman
pH 7 buffer. A titration curve of the standard buffer used in the growth medium was obtained and from this curve and the pH measurements of the culture supernatant the millimoles of acid per ml of culture produced in any given time period during the culture growth could be found. The initial pH of the culture was taken after aeration had begun and after inoculation but before growth had begun.

Residual ammonia nitrogen determinations were carried out by the method of Niss (1957) as adapted to this culture system by Ecker and Lockhart (1961b). All dilutions were made with distilled water and optical densities at 615 mp were determined against a distilled water-reagent blank.

Determinations of total carbohydrate material in the culture supernatant were based upon two methods. One was the anthrone method (Loewus, 1952) as modified by Ecker and Lockhart (1961b). This method had the disadvantage of being sensitive to many different carbohydrate materials, including the polymeric forms, so a second method based upon the Nelson's test for reducing sugars (Nelson, 1941) was also used. In the latter method 1 ml samples of culture filtrate, or a dilution thereof, were treated as described by Nelson and the optical densities at 750 mp were taken against a distilled water-reagent blank. Standard curves for both the anthrone and Nelson's tests are shown in Figure 2.
Figure 2. Standard curves for carbohydrate determination by anthrone test and Nelson's method

Open circles - Nelson's method (optical density at 750 μm)

Squares - anthrone test (optical density at 560 μm)
RESULTS

Effect of Initial Substrate Concentration
Upon Total Attainable Population

It was reported by Ecker and Lockhart (1961c) that, under conditions of a single limiting nutrient, the relationship between total attainable population in a culture and concentration of limiting nutrient can be expressed as:

\[ N_{\text{max}} = K'C_0^S \]  \hspace{1cm} (1)

where \( N_{\text{max}} \) is the maximum attainable population, \( K' \) is a constant, \( C_0 \) is the initial concentration of the limiting nutrient, and \( s \) is a second constant having a positive value less than one. A different form of Equation 1 is:

\[ \log_2 N_{\text{max}} = s \log_2 C_0 + \log_2 K' \]  \hspace{1cm} (2)

which can be recognized as the equation for a straight line having intercept \( \log_2 K' \) and slope \( s \). These two constants were ascertained to have characteristic values depending upon the strain of bacteria and the particular growth-limiting conditions.

The values for \( K' \) and \( s \) for the several strains, under carbon or nitrogen-limited conditions, are given in Table 2. The cells were grown in 10 ml volumes of medium in 18 x 150
Table 2. Values of $s$ and $\log_2 K'$ for the several mutant strains of *Escherichia coli* in both carbon- and nitrogen-limited media

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Nitrogen-limited</th>
<th>Carbon-limited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\log_2 K'$</td>
<td>$s$</td>
</tr>
<tr>
<td>KL2$^a$</td>
<td>25.2</td>
<td>0.558</td>
</tr>
<tr>
<td>KL2/G2</td>
<td>23.8</td>
<td>0.773</td>
</tr>
<tr>
<td>KL2/G3$^a$</td>
<td>26.1</td>
<td>0.546</td>
</tr>
<tr>
<td>KL2/G4</td>
<td>23.5</td>
<td>0.794</td>
</tr>
<tr>
<td>KL2/G5</td>
<td>22.4</td>
<td>0.885</td>
</tr>
<tr>
<td>KL2/G6$^a$</td>
<td>23.1</td>
<td>0.816</td>
</tr>
<tr>
<td>KL2/G7</td>
<td>22.8</td>
<td>0.816</td>
</tr>
<tr>
<td>KL2/G8$^a$</td>
<td>26.9</td>
<td>0.444</td>
</tr>
<tr>
<td>KL2/G9$^a$</td>
<td>26.1</td>
<td>0.573</td>
</tr>
<tr>
<td>KL2/G10</td>
<td>23.6</td>
<td>0.750</td>
</tr>
<tr>
<td>KL2/A4$^a$</td>
<td>22.4</td>
<td>0.919</td>
</tr>
<tr>
<td>KL2/TU2</td>
<td>23.9</td>
<td>0.797</td>
</tr>
<tr>
<td>KL2/N5</td>
<td>22.4</td>
<td>0.878</td>
</tr>
</tbody>
</table>

$^a$ $K'$ and $s$ values calculated from 30 or more points.

mm culture tubes aerated by bubble tubes (Lockhart and Ecker, 1958). OAR was not less than 40 m moles O$_2$/L/hr. The inoculum consisted of centrifuged and resuspended cells prepared as described in the Materials and Methods section.
The inoculum size, $G_0$ ($G_0 = \log_2 N_0 = \log_2$ number of cells/ml upon inoculation), varied between 22.5 and 23.5.

After 12 hrs of growth, by which time all cultures had attained stationary phase, samples were taken for total counts by the membrane filter technique. These counts were converted to their $G_{\text{max}}$ ($G_{\text{max}} = \log_2 N_{\text{max}}$) values and $K'$ and $s$ were calculated using the least squares method.

In its strict sense the relationship given in Equation 1 holds true only if the $N_{\text{max}}$ value used is equal to the total population found minus the inoculum value, $N_0$ (Ecker and Lockhart, 1961c). Table 3 gives the values of $N_{\text{max}}$, $G_{\text{max}}$, $N_{\text{max}} - N_0$ and $\log_2 (N_{\text{max}} - N_0)$ for the smallest total population used in calculating any given set of constants. In this particular instance the organism was K12/A4 and growth was limited at 50 $\mu$g ($\text{NH}_4 \text{SO}_4$/ml. The difference between $G_{\text{max}}$ and $\log_2 (N_{\text{max}} - N_0)$ values is negligible and becomes even smaller with increases in total population. Therefore $N_{\text{max}}$ was considered to be equivalent to $N_{\text{max}} - N_0$.

In Figure 3 representative plots of $G_{\text{max}}$ versus $\log_2 C_0$ ($C_0 = \mu$g ($\text{NH}_4 \text{SO}_4$/ml) are given. These strains represent the greatest variations found in $K'$ and $s$ values for nitrogen-limited systems. Examination of Table 2 indicates that carbon-limited systems show much less variation between strains in $s$ or $K'$ values.
Table 3. Comparison of various expressions of maximum population for *E. coli* K12/A4 in nitrogen-limited medium at the lowest total population values used in calculation of a given set of constants

<table>
<thead>
<tr>
<th>(N_{\text{max}})</th>
<th>(N_{\text{max}} - N_0)</th>
<th>(G_{\text{max}})</th>
<th>(\log_2(N_{\text{max}} - N_0))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.73 \times 10^8)</td>
<td>(1.64 \times 10^8)</td>
<td>27.4</td>
<td>27.3</td>
</tr>
<tr>
<td>(1.40 \times 10^8)</td>
<td>(1.31 \times 10^8)</td>
<td>27.1</td>
<td>27.0</td>
</tr>
<tr>
<td>(1.40 \times 10^8)</td>
<td>(1.32 \times 10^8)</td>
<td>27.1</td>
<td>27.0</td>
</tr>
</tbody>
</table>

It was often noted during cell counting and original isolation procedures that many of the mutant strains appeared to have smaller cells than the parent strain. This conclusion is also reached upon examination of the relationship between \(N_{\text{max}}\) and \(C_{\text{o}}\). If two strains are given the same initial concentration of limiting nutrient, and if the ratio of mass to volume is about the same for each, then the strain which produces the most cells must also produce smaller cells. In order to more closely examine this apparent cell size difference, and to obtain some semi-quantitative data on cell sizes for various strains, the cell measuring technique described in the section on Materials and Methods was employed. Comparative data for strains K12 and K12/G9 under nitrogen limitation are given in Table 4.

The data indicate that, in stationary phase in particular, K12/G9 does possess predominantly smaller cells than
Figure 3. Representative plots of $G_{\text{max}}$ versus $\log_2 C_0$ for four strains of *E. coli* in nitrogen-limited medium.

- Solid circles - K12/G8
- Open circles - K12
- Open triangles - K12/G6
- Open squares - K12/A4
Table 4. Cell size distributions for strains K12 and K12/G9 under nitrogen-limited conditions

<table>
<thead>
<tr>
<th>Cell length Whipple ocular units</th>
<th>Logarithmic phase</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K12</td>
<td>K12/G9</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>0.6</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>0.7</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>0.8</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>0.9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>1.1</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>1.2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

If the two strains were inoculated into a proper medium where each would have the same maximal rate of increase in mass, the parent strain would rapidly be outnumbered and overgrown by the mutant strain. This process is probably what occurred during the orthoselection procedure which produced K12/G9.

Similar data for cell size versus cell numbers were obtained for the other strains which had been intensively studied in both carbon- and nitrogen-limited systems. The
results were somewhat less striking in carbon-limited than in nitrogen-limited media. This is probably because the energy source is limited in the former case, whereas in the latter case there is enough energy available to enable the cells to divide as many times as their concentration of essential cellular components allows.

It is also apparent that (as indicated in Table 4) a bimodal distribution of cell sizes appears during growth in minimal medium. A possible explanation of this fact is that cell division and cell growth are not simultaneous processes under these conditions, but that cell division occurs over a given span of time and cell growth during a different time segment. Thus, if one considers one of the daughter cells of a cell having just undergone division there might be a period of time during which there is no evidence of increase in cell size, followed by a period of size increase which continues until the cell is about twice its original size, and then a second period of no size increase culminating in cell division.

Carbon-Limited Cultures

Cells for analysis were grown in 500 ml volumes of medium in straight-walled, one L bottles under the conditions of temperature and aeration previously described. The concentration of glucose was 800 µg/ml and $G_{max}$ ranged from
29.2 to 30.9 for the strains studied. Samples were taken aseptically at hourly intervals and centrifuged to remove the cells. The supernatant was removed and cells and supernatant were stored at \(-15^\circ C\) until analyses were completed. A typical plot of the resulting data is given in Figure 4. The strain represented here is K12/G8 and the limiting level of glucose is 800 \(\mu g/ml\) culture.

Carbon limitation of growth was verified by analysis for residual ammonia nitrogen in the culture filtrate. At the 15th hr the concentration of \(\left(\text{NH}_4\right)_2\text{SO}_4\) for all strains varied from \(2 \times 10^3\) to \(3.5 \times 10^3\) \(\mu g/ml\) culture.

It was observed that in all cases of carbon-limited growth the Nelson's test for reducing sugars indicated exhaustion of the glucose supply before the anthrone test. Both tests have a range of sensitivity from 2 to 20 \(\mu g/ml\) glucose when used as described. The anthrone test also indicated a low, but consistent, residuum of carbohydrate material ranging from 6 to 16 \(\mu g/ml\) culture, as glucose, after growth had ceased. The Nelson's test indicated no reducing sugar was present in stationary cultures.

This observation could be explained in either of two ways. One is that the glucose is converted to a nonreducing form, such as glucose-1-phosphate, before it is taken into the cell. The other explanation is based upon the wide range of materials to which the anthrone test is sensitive.
Figure 4. Plot of analysis data for strain K12/G8 in carbon-limited culture \( (C_o = 800 \mu g \text{ glucose/ml}) \)

- **Circles** - phenol protein
- **Squares** - biuret protein
- **Triangles** - extractable cellular nucleic acid
- **Solid circles** - acid production
- **Open arrow** - time at which no more glucose could be detected in the supernatant using Nelson's test
- **Solid arrow** - time at which only a small constant amount of carbohydrate could be detected in the supernatant using the anthrone test
These materials include polysaccharides as well as mono­
saccharides. The Nelson's test will indicate only the presence of a reducing end on a polysaccharide. An estima­
tion of the length of polysaccharides which possess a reducing end is possible by comparing the amount of carbo­
ydrate, as glucose, indicated by the anthrone test and the amount of glucose present as measured by a test for reducing sugars (Stewart and Nordin, 1961). If this method is applied to the carbon-limited culture data, and all the glucose present is assumed to be polymerized, the average chain would be 2 to 4 glucose units long. That E. coli produces polyglucose units of this chain length from maltose, lactose, glucose and glucose-1-phosphate has been demon­
strated previously (Monod and Torriani, 1949; Doudoroff et al., 1949; Palmstierna, 1955; Holme and Palmstierna, 1955).

It was also observed that the phenol protein concen­
tration in the cells was consistently lower than the biuret protein concentration after the cells had approached stationary phase. Both values, however, reach their peak at about the same time after glucose exhaustion. This discrepancy in total protein concentration between the two methods of analysis is possibly best explained by the dis­similar bases upon which these two procedures depend. The phenol test is sensitive to the presence of phenol groups,
Figure 5. Comparison of various characteristics of several strains of *E. coli* under carbon limitation ($C_0 = 300 \mu g$ glucose/ml)

- **Cell size** - average cell size, Whipple ocular units
- **Biuret protein** - $\mu g$ protein/ml culture
- **Phenol protein** - $\mu g$ protein/ml culture
- **Nephlos** - light scattering of a 1:20 dilution of culture
- **Nucleic acid** - $\mu g$ nucleic acid/ml culture

Strain designations are shortened ($K12/A4$ denoted by $A4$, etc.)
such as the tyrosine in protein, while the biuret test is based upon the color reaction of copper ions with the peptide bond. Therefore, variations in tyrosine concentration in the protein, inadequate solubilization of the protein, or interference by nonprotein groups which give the phenol reaction could restrict the precision of these tests.

Comparative values for various characteristics of strains K12, K12/A4, K12/G3, K12/G6, K12/G8 and K12/G9 in carbon-limited cultures are presented in Figure 5. These values are averages of the three highest hourly values occurring immediately after glucose exhaustion. None of the values used to calculate the averages varied from the highest value by more than 20% and most varied less than 10%. The significance of their relationships will be discussed later.

Nitrogen-Limited Cultures

It is evident from Table 2 that K' and s are more variable for nitrogen-limited than carbon-limited cultures, and if both K' and s vary for two strains then there should be an intersection of their plots of $G_{\text{max}}$ versus $\log_2 C_0$. The value of $\log_2 C_0$ at which $G_{\text{max}}$ is most nearly the same for the diverse strains is about 9.5. Therefore the nitrogen-limited cultures were limited at $C_0 = 800 \mu g (NH_4)_2SO_4/ml (\log_2 C_0 = 9.62)$ which produced a population
sufficiently high to be easily handled analytically and obviated the possibility of error in interstrain comparisons due to wide differences in size of populations. \( G_{\text{max}} \) ranged from 30.4 to 31.5 for the strains studied under these conditions.

Cells were grown under the same conditions as carbon-limited cultures as far as growth and sampling procedures were concerned. Residual glucose samples were taken throughout the greater part of the growth curve. The residual glucose values obtained varied widely, probably because of inaccuracies in the dilution process necessary to reach the proper concentration range for analysis. The concentrations of glucose at the time sampling was discontinued ranged from \( 4 \times 10^3 \) to \( 1.1 \times 10^4 \) \( \mu g/ml \). This verifies that the conditions were nitrogen- rather than carbon-limited.

A typical plot of the resulting data for the parent strain, K12, is given in Figure 6.

Several general differences between carbon-limited and nitrogen-limited cultures can be seen in the comparison of Figures 4 and 6. In all strains the amount of phenol protein is greater than the amount of biuret protein in nitrogen-limited cultures, whereas in carbon-limited cultures this relationship is reversed.

In several strains the increase in biuret protein, phenol protein, and light scattering continued after the
Figure 6. **Data for Escherichia coli K12 under nitrogen-limited growth conditions** \( (C_0 = 600 \mu g \ (\text{NH}_4)_2\text{SO}_4/\text{ml}) \)

- Open circles - phenol protein
- Squares - biuret protein
- Triangles - extractable cellular nucleic acid
- Solid circles - acid production
- Arrow - time of nitrogen exhaustion or time when no more ammonia nitrogen could be detected in the culture filtrate by analytical procedures used
nitrogen supply had been exhausted. Variations between strains were observed and in some cases only one or two of these parameters continued to increase after nitrogen exhaustion while the others remained constant or decreased slightly.

The continued production of acid after nitrogen exhaustion, found in all strains, is contrary to the report of Ecker and Lockhart (1961b). This contradiction may be explained, however, by the difference in level of non-limiting substrate between the two sets of data. The residual glucose was always in abundant supply in the experiments reported here but was consumed within two hours after nitrogen exhaustion in the data reported by Ecker and Lockhart.

The time of nitrogen exhaustion in nitrogen-limited cultures was found to be much more variable than the time of glucose exhaustion in carbon-limited cultures. Glucose exhaustion occurred from 5 to 8 hours after inoculation but the time of nitrogen exhaustion varied from 8 to 13 hrs after inoculation. An error in $G_0$ values of plus or minus one log unit could explain the variation in times of glucose exhaustion but not in times of nitrogen exhaustion.

The latter variation may be accounted for by the uses to which glucose and nitrogen are put during growth. It has been suggested (Ecker and Lockhart, 1961c) that glucose
Figure 7. Comparison of various characteristics of several strains of E. coli under nitrogen limitation ($C_0 = 600 \mu g (NH_4)_2SO_4/ml$)

- **Cell size**: average cell size, Whipple ocular units
- **Phenol protein**: $\mu g$ protein/ml culture
- **Nucleic acid**: $\mu g$ nucleic acid/ml culture
- **Acid level**: $\mu$ moles acid/ml at time of nitrogen exhaustion
- **Acid rate**: rate of acid production, $\mu$ moles/ml/hr, after nitrogen exhaustion

Strain designations are shortened ($\text{K12/A4}$ denoted by $\text{A4}$, etc.)
<table>
<thead>
<tr>
<th>$K'$ \times 10^{-6}$</th>
<th>S</th>
<th>Cell Size</th>
<th>Phenol Protein</th>
<th>Nucleic Acid Level</th>
<th>Acid Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>103.0</td>
<td>0.44</td>
<td>0.77</td>
<td>475 G9</td>
<td>170 G6</td>
<td>19.0 G3</td>
</tr>
<tr>
<td>83.5</td>
<td>0.54</td>
<td>0.75</td>
<td>432 G3</td>
<td>164</td>
<td>17.6 G8</td>
</tr>
<tr>
<td>64.0</td>
<td>0.64</td>
<td>0.73</td>
<td>389 A4</td>
<td>158 G9</td>
<td>16.2 G9</td>
</tr>
<tr>
<td>44.6</td>
<td>0.73</td>
<td>0.72</td>
<td>346 K12</td>
<td>152 K12</td>
<td>14.8 G8</td>
</tr>
<tr>
<td>25.1</td>
<td>0.82</td>
<td>0.71</td>
<td>303 G6</td>
<td>146 A4</td>
<td>13.4 K12</td>
</tr>
<tr>
<td>5.6 A4</td>
<td>0.92</td>
<td>0.69</td>
<td>260 K12</td>
<td>140 A4</td>
<td>12.0 G6</td>
</tr>
</tbody>
</table>
is used in two, and perhaps three, basic processes during growth. Nitrogen would be used only for synthesis, since it cannot serve as an energy source. Therefore those strains which may be more efficient in nitrogen utilization for synthesis would exhaust the nitrogen supply later than those strains less efficient.

Comparative values for measured characteristics of strains K12, K12/A4, K12/G3, K12/G6, K12/G8 and K12/G9 are presented in Figure 7.
DISCUSSION

The transition of a bacterial culture to stationary phase from a rapidly growing system, increasing in cellular mass and numbers at a maximal rate, is sometimes considered a relatively simple procedure. The literature of bacterial growth abounds with attempts to define a single growth-controlling reaction. These attempts are based on the concept that bacterial growth is primarily autocatalytic in nature; i.e., as cellular mass or cell numbers increase, the rate at which nutrients are used to produce new cells increases proportionally. The transition phase in such a system would be a smooth, though relatively sudden cessation of all reactions in the system when the supply of raw materials was exhausted. The stationary phase cells would be metabolically analogous to the exponentially growing cells from which they arose excepting that production of cellular material had ceased as a result of substrate exhaustion.

A more correct representation of a bacterial culture in transition probably would show a series of chemical reactions approaching various equilibria at changing rates, each influenced by those metabolically preceding it and influencing those after it in sequence. Thus, the transition phase may have its origins in enzymatic and chemical events which
occur before any obvious manifestations of transition appear. The overall result would be a system in which various reactions take precedence at different times in the metabolic operation of the culture, with biochemical variations which occur over a span of time encompassing, but not limited to, the transition phase. Differences between strains in emphasis upon various reactions could also result in variations in growth constants such as $K'$ and $s$.

Changes in cell content and culture characteristics, indicating the existence of such an interacting system of chemical equilibria, are observed to occur before, during and after the entrance of the culture into stationary phase. This process of biochemical differentiation could be triggered by several factors such as impending nutrient exhaustion, change in content of a particular nutrient on a per cell basis, or changes in relative concentrations of a series of nutrient materials (Lockhart, 1959).

Ecker and Lockhart (1961c) have indicated that appreciable changes in rates of glucose utilization take place in cultures of *Escherichia coli* K12 which are subjected to oxygen-limiting conditions. These changes occur before the end of logarithmic growth becomes apparent. If the processes of biochemical adaptation to low oxygen availability can occur before the processes responsible for increase in cell numbers show the effects of oxygen depletion, then
perhaps one would expect similar effects before depletion of other growth-limiting substrates.

The levels of certain constituents of the cells and culture filtrates were plotted against the total amounts of growth-limiting substrate used by the cells. This was done for only the nitrogen-limited systems since the concentrations of reducing sugar and total carbohydrate in glucose-limited cultures differ by about the same ratio at all times during culture growth. The plots of phenol protein production and cellular nucleic acid production versus the amount of \((\text{NH}_4)_2\text{SO}_4\) used up to that point are given in Figures 8 and 9 for strains K12, K12/A4, K12/G3 and K12/G8. These two figures indicate that, as growth progresses and the concentration of available nutrient per cell decreases, changes occur in either the efficiency or mechanisms of production of cellular components from nutrients. These changes, though qualitatively similar, differ quantitatively between strains and could be due to differences in chemical equilibria or in feedback controlled alterations in reaction rates leading to a process of biochemical differentiation unique for each strain.

Some concept of the nature of the competing metabolic processes involved in these shifts of biochemical emphasis may be gained by inspection of the events occurring during
Figure 8. Production of phenol protein versus the amount of $(\text{NH}_4)_2\text{SO}_4$ used by cultures limited at $C_0 = 600 \mu g (\text{NH}_4)_2\text{SO}_4/ml$

- Open circles - strain Kl2
- Solid circles - strain Kl2/A4
- Squares - strain Kl2/G3
- Triangles - strain Kl2/G8
Figure 9. Production of cellular nucleic acid versus the amount of \((\text{NH}_4)_2\text{SO}_4\) used by cultures limited at \(C_0 = 600 \mu g (\text{NH}_4)_2\text{SO}_4/\text{ml}\)

- Open circles - strain K12
- Solid circles - strain K12/A4
- Squares - strain K12/G3
- Triangles - strain K12/G6
the period of transition. For example, in glucose-limited systems the reducing sugar component of the carbohydrate material in the medium disappears before the total carbohydrate present is exhausted. Unlike the total carbohydrate, which shows a reasonably constant concentration remaining after growth has ceased, the reducing sugar disappears completely by the beginning of the stationary phase. The difference in concentration of the two materials found in stationary phase cannot be attributed to a difference in sensitivity of the tests since they are both accurate in the range of 2 to 20 µg glucose.

The precise nature of the carbohydrate materials in the culture medium after the reducing glucose has disappeared is not known at present. Ascending paper chromatograms show only spots corresponding in \( R_F \) values with glucose and faint spots close to the origin indicating higher molecular weight carbohydrates or other compounds. The solvent used in this work was 80% propanol and the chromatograms were developed using the diphenylamine reagent (Buchan and Savage, 1952) or the silver nitrate dip (Youngquist, 1960). The latter method consists of dipping the dry chromatogram first into a dilute solution of silver nitrate in acetone and then into basic methanol. The silver nitrate reagent is sensitive to small amounts, about 5 µg, of reducing sugars.
It was also noted that the pH increases somewhat after growth has ceased in glucose-limited cultures. This pH increase, in the work reported here, corresponds to the removal of from two to four micromoles of acid from each ml of culture. It has been reported (Roberts et al., 1955) that acetate and CO₂ are the primary extracellular products of aerobic glucose metabolism by E. coli. With glucose as sole carbon source for an aerobic culture, 15% of the glucose appeared as acetate. Using this figure, a culture provided with 800 µg of glucose would produce 120 µg of acetate. The pH rise observed would necessitate the reabsorption of about 200 µg of acetic acid and could be attributed to the reabsorption of the organic acids produced by the cells and excreted into the medium. However, the total pH drop in the culture medium cannot be attributed only to the production of organic acids and must indicate some other effect of the biochemically active bacteria upon the culture medium.

If it is assumed that at least a portion of the rise in pH values after transition is due to the reutilization of organic acids previously produced, then it is possible to resolve the utilization of carbon source into three divisions. The first of these consists of both direct utilization of the reducing glucose in the medium and its conversion to some material which is carbohydrate but nonreducing in
character. The second phase is the utilization of all but a small portion of this nonreducing carbohydrate. The third phase is the reabsorption and utilization of the organic acids in the medium which are amenable to this process. Only after this process is complete has the culture become truly carbon-limited.

In nitrogen-limited cultures, the times of nitrogen exhaustion vary from strain to strain. If the process of exhaustion of substrate nitrogen is considered to consist of several phases, as was postulated in the case of carbon limitation, the differences in time can be explained as modifications in the rates of assimilation and utilization by cells of the different strains. The first such process would be the passage of the ammonium ion through the bacterial cell wall and its incorporation into some compound such as glutamate or aspartate. Differences among strains at this stage may indicate differences in cell permeability. The second stage would be utilization of the initially produced nitrogenous compounds for synthesis of cellular components. The organism which had rapid assimilation and normal or slow synthesis would show an early exhaustion of ammonium ions and would continue to synthesize nitrogenous cellular components after the nitrogen in the medium was exhausted. The organism which had slow assimilation and normal or rapid synthesis would show a late exhaustion of
ammonium ion and would reach its peak protein content coincident with or shortly after the point of nitrogen exhaustion.

This sequence of events can be demonstrated for strains K12/G6 and K12/G6 under nitrogen-limited conditions. K12/G6 shows exhaustion of nitrogen, as ammonium ion, in the medium between the 8th and 9th hour of growth in medium containing 600 μg/ml (NH₄)₂SO₄ but does not reach its peak value in biuret protein until between the 10th and 11th hour. Strain K12/G6, under similar culture conditions, shows exhaustion of ammonium ion between the 12th and 13th hour but also reaches its peak value in biuret protein at the same time, or even slightly before this time.

In nitrogen-limited systems a constant rate of acid production is found after the stationary phase has been reached. The initiation of this arithmetic increase in acid content with time appears to be coincident with nitrogen exhaustion for all strains studied. Figure 10 demonstrates the differences in acid production before and after nitrogen exhaustion for three of the strains. It will also be noticed from this figure that strains which produce the most acid before and after nitrogen exhaustion also have the latest times of nitrogen exhaustion.

A comparison between numbers of cells, cellular mass and rate of acid production after nitrogen exhaustion is
Figure 10. Differences in quantities of acid produced and rates of acid production for several strains of *E. coli* under nitrogen-limited conditions ($C_0 = 600 \mu g (NH_4)_2SO_4/ml$)

Circles - strain K12/A4 (nitrogen exhaustion between 9th and 10th hr of incubation)

Squares - strain K12/G3 (nitrogen exhaustion between 12th and 13th hr)

Triangles - strain K12/G8 (nitrogen exhaustion between 12th and 13th hr)

Arrow - point at which no more ammonia nitrogen could be detected in the culture filtrate
ACID PRODUCTION \( \mu \text{moles} / \text{ml culture} \)

HOURS BEFORE AND AFTER NITROGEN EXHAUSTION
Table 5. Rate of acid production after nitrogen exhaustion related to cellular mass and cell numbers for several strains of *E. coli*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of acid production after nitrogen exhaustion</th>
<th>Biuret protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micromoles/ml/hr</td>
<td>Micromoles/µg</td>
</tr>
<tr>
<td>K12/G3</td>
<td>2.33</td>
<td>6.47 x 10^{-3}</td>
</tr>
<tr>
<td>K12/G9</td>
<td>1.80</td>
<td>3.95 x 10^{-3}</td>
</tr>
<tr>
<td>K12/G8</td>
<td>1.57</td>
<td>4.36 x 10^{-3}</td>
</tr>
<tr>
<td>K12</td>
<td>1.20</td>
<td>3.35 x 10^{-3}</td>
</tr>
<tr>
<td>K12/G6</td>
<td>1.08</td>
<td>2.18 x 10^{-3}</td>
</tr>
<tr>
<td>K12/A4</td>
<td>0.72</td>
<td>1.55 x 10^{-3}</td>
</tr>
</tbody>
</table>

shown in Table 5. It appears that the type of protein found in the various strains differs qualitatively as well as quantitatively. The levels, per unit bacterial mass, of the enzymes responsible for pH changes in the medium must be quite different for the various strains.

The effect of the pH of the medium upon the metabolism of the cells and upon the total population attainable is complex. Hinshelwood (1946) has indicated that pH may have definite effects upon the toxicity of accumulated metabolic products. Jordan and Jacobs (1948) noted that the effect of pH on the level of population attainable varies with temperature of incubation.
Other investigators have observed effects of pH upon specific aspects of cell metabolism. Ingram (1940) found the pH optimum for respiration of *E. coli* to be about 7.3; Kipper (1952) ascertained that the pH optimum for reduction of 2,3,5-triphenyltetrazolium chloride was 7.5 for cells of *E. coli* four hours after inoculation and 6.5 for cells 18 hrs old. Toennies and Shockman (1961) determined that cells of *Streptococcus faecalis* grown in lysine limited media were much less sensitive to "depletion lysis" when grown at pH 5.9 than at pH 6.5.

At any rate, the process of biochemical differentiation (accompanied by changes in amounts of various materials in the cells) continues after growth has ceased. Figures 4 and 6 indicated some of the fluctuations occurring in carbon-limited and nitrogen-limited systems, respectively. In general, in the carbon-limited systems, the peaks reached in biuret protein, phenol protein and cellular nucleic acid are followed by a rapid decrease in these values and then by a secondary peak. The initial drop in values of the protein compounds corresponds with an increase in the nucleic acid content of the culture filtrate in all cases except K12/36. This would indicate that a period of lysis of cell "leakage" occurs a short time after the stationary phase has been reached. This is easily understood for carbon-limited cultures if part of the energy derived from the carbon
source had been used in some dynamic fashion to "sustain" the cell as an integral unit.

In nitrogen-limited cultures the situation after stationary phase has been reached is different from that in carbon-limited cultures. In the former the energy source is in excess and the integrity of the cell can be more easily maintained, if energy is required for this purpose. Despite this, however, the same general pattern as was described for carbon-limited cultures is found in nitrogen-limited after stationary phase has been reached.

The identity of the material found in culture filtrates under these conditions has been investigated by Dagley and Johnson (1956). They observed that amino acids and peptides appeared in the culture filtrate during growth of *E. coli* and other bacterial species in aerated minimal salts medium. They also found that the pattern of amino acids and peptides in the filtrate was reproducible for a given organism at a defined point of growth on a particular carbon source. It was hypothesized that the amino acids and peptides appeared in the filtrate because their rate of synthesis was greater than their rate of utilization.

In the work discussed here no attempt was made to identify the materials in the culture filtrate. It was found, however, that in nitrogen-limited media K12 and the G series of mutants could be divided into two general groups
on the basis of the amount of protein appearing in the filtrate and its time of appearance in relation to nutrient exhaustion. Figure 11 shows the hourly levels of filtrate protein for three strains in carbon-limited and in nitrogen-limited media. K12, K12/G3 and K12/G8 all have relatively late times of nitrogen exhaustion and show large and generally increasing amounts of protein in the filtrate. The opposite situation exists in the case of K12/G6 and K12/G9. The same general strain patterns of filtrate protein content are shown in the carbon-limited situation.

The recent work of Quinn and Biggs (1961) has indicated that strains K12 and K12/N5 show different X-ray diffraction patterns for certain fractions of dried, cell-free preparations. Presence of distinct crystalites in the dried preparations may indicate variations in the ability of the two strains to concentrate materials from the medium within their cells, and consequently, differences in permeability or active transport systems. It might be informative to obtain such data for strains differing as widely in lysis or cell "leakage" as K12, K12/G6 and K12/G8.

Up to this point in the discussion no attempt has been made to interrelate more than perfunctorily the various constants and growth characteristics of the strains studied. If the values of $K'$ and $s$ are to have meaning with reference to bacterial growth they must be related in some manner to
Figure 11. Hourly levels of protein in culture filtrate for various strains under carbon- and nitrogen-limited conditions

A. Carbon-limited culture ($C_0 = 800 \mu g$ glucose/ml)
- Solid circles - strain K12
- Solid squares - strain K12/G8
- Solid triangles - strain K12/G6

B. Nitrogen-limited culture ($C_0 = 600 \mu g$ $(NH_4)_2SO_4/ml$)
- Open circles - strain K12
- Open squares - strain K12/G8
- Open triangles - strain K12/G6
- Arrow - point at which no more ammonia nitrogen could be detected in the culture filtrate
the cellular and growth characteristics of the respective strains. The relationships found here, though qualitative in nature and subject to revision, at least indicate the more fertile areas for future investigation.

It may be profitable to examine the theoretical meanings of $K'$ and $s$ before describing the relationships found. $K'$ is the theoretical maximum population reached when the initial concentration of the growth-limiting substrate is 1 $\mu$g/ml and the inoculum is one cell/ml. It may be described as the efficiency of the strain in producing cells from substrate. The constant $s$ may be defined as the ability of the strain to maintain this efficiency of substrate utilization with increasing initial concentrations of substrate.

In order to compare these values with the growth characteristics of the various strains the data in Figures 5 and 7 may be considered again. In the case of carbon limitation (Figure 5) it appears that $K'$ and $s$ are directly related; with the single exception of strain K12/G6, if $K'$ is low in value then $s$ is also low. $K'$ and $s$ values appear to be inversely related to cell size and to the amount of protein appearing in the culture filtrate; i.e., when $K'$ or $s$ are high in value the cells are short and the amount of protein appearing in the culture filtrate is small. The constants are also related to the increase or decrease in
light scattering values after the exhaustion of the substrate. In the cases of K12 and K12/A4, where the values of both $K'$ and $s$ are low, the light scattering values increase, whereas when either $K'$ or $s$ is high the light scattering values tend to decrease after substrate exhaustion. In summary, in carbon-limited cultures the values of $K'$ and $s$ appear to be directly related to each other and inversely related to cell size, to the amount of protein appearing in the culture filtrate and to increase in light scattering after exhaustion of the carbon source.

The relations of $K'$ and $s$ in carbon-limited cultures to the amount of biuret protein, phenol protein and extractable nucleic acid in the cells appears to be complicated by secondary relationships among the three analytical values for any given strain. If one of these is high in a given culture the other two are likely to be medium or low in value.

As was shown in Table 2, the values for $K'$ and $s$ with carbon as the limiting substrate did not differ widely among strains, so that striking variations in other growth characteristics would perhaps not be expected for carbon-limited cultures. Under nitrogen limitation, on the other hand, markedly different values for both $K'$ and $s$ were obtained for the various strains. When comparisons are made between strains in nitrogen-limited systems (Figure 7),
the values of $K'$ and $s$ appear to be inversely rather than directly related to each other. $K'$ values are directly related to the amount of phenol protein produced by the culture, the amount of acid produced by the culture at the time of nitrogen exhaustion and to the rate at which acid is produced by the culture after nitrogen exhaustion. The time of nitrogen exhaustion appears to be directly related to the $s$ value; i.e., strains having low $s$ values appear to deplete the nitrogen at a lesser rate than those having high $s$ values and, consequently, to have later times of nitrogen exhaustion.

These indications of the relationships existing between $K'$ and $s$ values and the various characteristics of growth are sufficient to allow speculation as to what these constants represent and what their biological significance may be. The constant $K'$ appears to be a measure of the efficiency of the various strains in production of cells from substrate. If the strains are placed in order of increasing $K'$ values we find that the order for glucose-limited cultures is $K_{12/G6}$, $K_{12/A4}$, $K_{12}$, $K_{12/G3}$, $K_{12/G8}$ and $K_{12/G9}$. For nitrogen-limited cultures the order is the same except that the positions of $K_{12/G6}$ and $K_{12/A4}$ are reversed and the positions of $K_{12/G8}$ and $K_{12/G9}$ are reversed. The observation that $K'$ is essentially independent of the nature of the nutrient material and the fact that the strains have
substantially the same ranking with respect to one another, regardless of whether carbon or nitrogen is growth-limiting, suggests that the relative value of $K'$ is less dependent upon the nature of the limiting substrate than upon some genetic characteristic of the particular strain. This characteristic could be a tendency of some strains to divide immediately, as soon as enough cell material is present, to provide two viable daughter cells. General corroboration of this hypothesis is indicated by comparison of $K'$ values and average cell sizes in both nitrogen- and carbon-limited media. Strain K12/A4, possessing a low $K'$ value, produces cells whose average size, in either carbon- or nitrogen-limited media, is relatively large, whereas K12/G9, possessing high $K'$ values under either type of limitation, consistently produces cells whose average size is smaller than those of any other strain.

The constant $s$ appears to be of a different intrinsic nature than $K'$. Apparently there is little correlation between the patterns of physiological characteristics associated with high or low values of $s$ in carbon-limited versus nitrogen-limited systems. The values of the constant $s$ under nitrogen-limited conditions vary widely but in a fairly predictable manner. With the exception of strain K12 the low values of $s$ correspond with high $K'$ values, high concentrations of acid in the medium at the time of
nitrogen exhaustion, high rates of acid production after nitrogen exhaustion and relatively late times of nitrogen exhaustion. In carbon-limited systems s varies much less among strains than the corresponding constant in nitrogen-limited systems. In this case s is directly related to K' and inversely related to cell size, to amount of protein in the culture filtrate and to increase in light scattering after protein exhaustion.

In nitrogen-limited cultures the inverse relationship between s values and acid production for the various strains suggests that retention of efficiency in utilization of nitrogen may depend on acid production and the subsequent effect of the resulting pH in the medium upon the metabolism of the cells.
CONCLUSIONS

During the process of orthoselection of the mutants studied here, populations of the parent strain, Kl2, were subjected to selective environments which favored the survival of any mutants better adapted to the artificial culture system imposed. While it is not possible to define fully the selective advantages possessed by the mutants, their primary differences from the parent strain appear to be in total population achieved per unit available nutrient, in ability to maintain this efficiency of cell generation at larger concentrations of the limiting substrate and, possibly, in ability to retain essential materials within the cell. These variations have resulted in differences among strains in the values of the growth constants $K'$ and $s$.

Comparison of the closely related strains described here allows the statement of three general conclusions. The first is that the changes in metabolism and biochemical characteristics which are typical of the transition phase between exponential growth and stationary phase begin to occur, or are set in motion, before the exhaustion of the limiting nutrient occurs. The second is that this process of biochemical differentiation is not simple in nature but appears to consist of a series of competing and interrelated
changes in the emphasis that the cell's metabolic system places upon individual reactions and reaction sequences. These changes have distinctive patterns which are quantitatively different between strains and qualitatively distinctive for particular limiting nutrients. The third conclusion is that it is possible to relate the values of the growth constants $K'$ and $s$ to the growth characteristics of particular strains and partially to elucidate the biological significance of these constants by relating them to characteristics which are comparable between strains.

The values of $K'$ for the various strains appear to be determined by a genetic modification in the efficiency of the metabolic process leading to cell division. $K'$ values are directly related to high phenol protein production and high acid production in nitrogen-limited systems and to low filtrate protein concentration in carbon-limited systems. The value of $K'$ is inversely related to cell size; i.e., the larger the value of $K'$ the smaller is the average cell size in both carbon- and nitrogen-limited systems. High acid production under nitrogen limitation or the smaller cell sizes may provide markers for future genetic studies of the mutant strains.

The values of $s$ apparently denote a different culture characteristic than do the values of $K'$. The two are directly related in carbon-limited systems, where $s$ varies
only slightly between strains, and inversely related in nitrogen-limited systems. The values of s appear to be directly related to the amount of acid produced in cultures which are nitrogen-limited and may be indicative of a metabolically produced change in the nature of the culture medium with subsequent effects upon efficiency of growth. Future work under conditions of controlled pH during growth might define this relationship more closely.

It might now be possible to further clarify the process of biochemical differentiation occurring in these mutants, and thus to investigate the nature of genetic control of growth characteristics. Modification of the sampling techniques used here, in order to give samples at smaller than hourly intervals, and separate analysis for DNA and RNA content per cell for the various strains, would assist in achieving this objective. Identification of materials discharged into the culture medium would indicate metabolic changes occurring during biochemical differentiation and might provide markers for genetic exploration.


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