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Constancy of Growth on Simple and Complex Media

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An apparatus has been developed in which bacterial growth can be measured very precisely over short intervals of time. Its precision is presented and used to assess the constancy of growth in batch culture. Under certain conditions, i.e., Luria broth or 0.2% glucose-M9 medium at very low cell densities, the specific growth rate of *Escherichia coli* appeared to be constant within the measurement limits of the method. In succinate minimal medium, the growth rate increased gradually over several days and never became constant. With nutrient broth and with Luria broth, growth slowed progressively at moderate cell densities within the range considered to be in the logarithmic phase of growth. In addition, temporary slowdown in growth rate occurred in these two complex media at characteristic cell densities. These gradual increases in succinate minimal medium and temporary slowdowns in the complex media would be undetectable without precise measurements and may have been a source of variability in many bacteriological studies.

We have been developing a method to follow growth of a bacterial culture which enables both continuous and very precise measurements. We employ a Cary model 16 double-beam spectrophotometer linked to a Wang 720 C mini-computer (1, 4). In the current studies, an increase in sensitivity of the apparatus has been achieved with a long-light-path (10 cm) flow-through cuvette that permits simultaneous stirring and aeration without introducing air bubbles and turbulence into the light path.

This apparatus has been used for a variety of purposes, such as to study the kinetics of drug penetration and inhibition of growth and the detailed kinetics of shift-up experiments, and to measure substrate dependence for growth. These studies are the subject of other papers. The present paper describes the apparatus and details its precision. This paper also examines systematic and nonsystematic fluctuations in specific growth rate in complex and minimal media.

This approach to the study of growth physiology depends on the finding (3) that turbidity, measured with a narrow-beam spectrophotometer and corrected for Beer's law deviation, is related to the concentration of solids with the same proportionality constant for cell size over an eightfold range that encompasses the smallest to the largest of the cells in the cultures studied here. In the absence of clumping and when the water concentration of the cells does not change, the slope of plot of the logarithm of the dry-weight concentration (inferred from the turbidity) versus time is an accurate measurement of the specific growth of biomass. It is also the specific growth rate of cell numbers in cases where the average cell size is constant.

MATERIALS AND METHODS

Growth conditions were those previously employed (4). *Escherichia coli* ML308 was used throughout. Overnight cultures, limited by 0.02% succinate or glucose, were stored in the refrigerator and used to start the working cultures that were then used to fill the cuvette system.

The growth measurements were made as indicated previously (1, 4) with further minor improvements in computer software. The major improvement was the utilization of a flow cell in which the mixing and aeration are conducted in such a way as to reduce the fluctuations in turbidimetric measurement (Fig. 1) below those of our previous magnetically stirred apparatus (1). With the long light path we could work with lower concentration of cells and, therefore, with decreased depletion by the organisms of nutrients or the formation of inhibitors. Thus, there was less chance of cell-cell interaction. The same stirring and aeration principle was employed with a 1-cm flow-through cuvette. The cuvettes were filled with water and then ethanol between runs to clean and sterilize them. The growth measurements were of short enough duration so that aseptic conditions were not necessary.

Briefly, the apparatus was operated as follows. The jacket of the 10-cm cuvette and the cuvette chamber were maintained at 37.0 ± 0.01°C in a Cary model 16 double-beam spectrophotometer. The 1-cm cuvette was placed in a sample holder made of brass in contact with thermostated water. The growth run was initiated by taking 1 ml from a culture that had been maintained by dilution for at least 20 generations in active growth

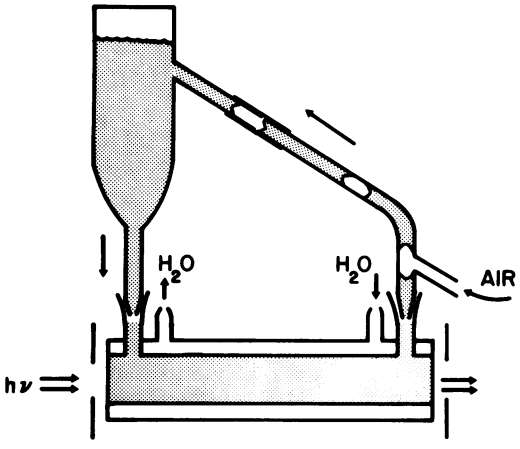

FIG. 1. *Cuvette system to measure bacterial growth on-line. The jacketed quartz cuvette has a 10.000-cm light path. The reservoir and air inlet are glass with connecting rubber tubing. Air is supplied from an aquarium air pump saturated with water by passage through an air bubbler. The air not only aerates the culture but causes the circulation of the bacterial culture through the system about once every 12 s. The mixing transient of the apparatus is 98% complete in 30 s. The cell is of quartz and is a Hellma, Inc. (Jamaica, N.Y.) no. 165-QS cell specially modified so that the input and output connections are closer to the ends. The 1-cm cuvette system is operated analogously; the cuvette itself is a Hellma no. 154-QS.*

at low cell densities. This was added to 39 ml of the same kind of fresh medium previously placed in the growth chamber. One-tenth these amounts was used for the 1-cm system. Care was always taken to preequilibrate the solutions at the growth temperature.

Three measurements of the apparent absorbance at 660 nm or 420 nm were made every 2 s. Measurements accumulated over each 2 s for 200-s intervals (or 12 s for 20-min intervals) were averaged and stored. After 100 measurement intervals, each average was retrieved and converted to a dry-weight concentration with a formula that corrects for deviations from Beer's law (3). For the 1-cm cuvette system with 660 nm light, the dry-weight concentration is given by: $W = 1293.1 (1 - \sqrt{1 - 0.60881A})$ μg/ml, where A is the absorbance. Then the linear regression of ln W against time for the 100 data points was carried out by the computer to fit the equation: $\ln W = \ln W_0 + \lambda t$. The results were typed out by the Wang IBM output typewriter, and measurements of turbidity were resumed to initiate a new measurement interval. The output includes W_0, the bacterial concentration at the beginning of the interval calculated from the intercept of the regression, and the specific growth rate, λ, calculated from the slope of the regression.

The computer output also includes the coefficient of variation of the specific growth rate, sd_λ, a statistic which has been of help during the actual experiment as well as when interpreting data. If the aeration was erratic or some other experimental problem arose, it was usually first detected by high and erratic fluctuations of this coefficient. The standard deviation of the slope of data fitted to the linear relationship $Y = a_0 + a_1X$ is given by:

$$sd_\lambda = sd_{a_1} = \sqrt{\frac{(\Sigma Y^2 - a_0\Sigma Y - a_1\Sigma XY)n}{(n-2)[\Sigma X^2 - (\Sigma X)^2/n]}}$$

In our case, the Ys are the natural logarithms of the dry-weight concentration, (ln W), the Xs are the times, a_0 is the intercept, a_1 is the slope, and n is 100.

The long-term photometric stability of the Cary spectrophotometer was such that the balance changed no more than 0.002 A at 660 nm in a 24-h period. For precision measurement of growth rate, a constant air stream is critical to achieve both adequate stirring and aeration. However, the range of flow achieving satisfactory aeration is fairly large. Two bubbles per second was used in the present studies. Air flow from an aquarium pump, bubbled through a water bottle, was controlled by a micrometer valve. During aerations a few very small air bubbles entered the light path of the 10-cm cuvette system, resulting in an absorbance increase of about 0.003 A. The increase was less in the 1-cm cuvette system. The effective noise in the system due to photometric and electronic noise as well as air bubbles (converted to the same units [h^{-1}] as the specific growth rates) varied with the absorbance due to a nonturbid dye solution, but corresponded to 1 ± 0.05 standard deviation per h at the low end of the range of turbidities employed between $A = 0.01$ and $A = 0.1$. The effective noise was about ±0.01/h in the middle of the range and about ±0.002/h when the turbidity was such as to yield an absorbance value near 1.

These sources of variability do not include errors due to turbidity fluctuations. The latter were assessed by following cultures that were nongrowing due to lack of a carbon source. Conditions were chosen where there was no long-term change in turbidity (no evaporation, no lysis, etc.) At a turbidity corresponding to 0.5 A, the fluctuation of turbidity within a 200-s interval of measurements led to an error corresponding to sd_λ of ±0.02/h in the 10-cm system. Thus in this range the turbidity fluctuations are comparable with all other sources of error. Of course, all these random errors can be reduced by increasing the length of the interval of measurement or by pooling data from successive 200-s intervals of measurement. Note that, if by chance an air bubble were to lodge in the optical path, or any equivalent discontinuous change were to occur, this would cause an erroneous estimate of λ, but would not cause significant error in the succeeding values of λ.

RESULTS

Growth on complex media. We followed the growth of a number of (in principle) identical cultures from a single liquid culture stored in the refrigerator, subcultured in the same medium (Difco nutrient broth) under the same conditions from a very small inoculum within both the 10- and 1-cm growth chambers (Fig. 2). Successive λ values of the specific growth rate

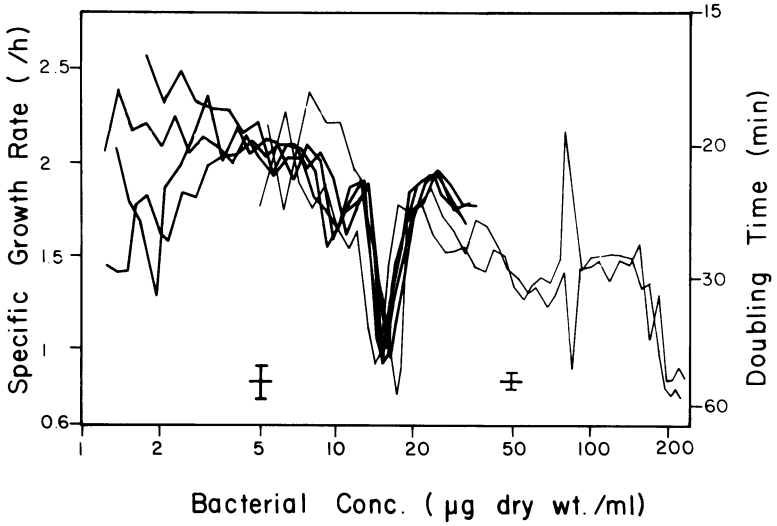

FIG. 2. *Growth of six nutrient broth cultures of E. coli ML308 in the computer-linked spectrophotometer at 37.0°C. The specific growth rates estimated from successive 200-s intervals are presented as a function of the dry weight concentration, given as abscissa. The right-hand ordinate gives the corresponding doubling times from* $T_2 = (\ln 2)/\lambda$. *Measurements in the 10-cm cuvette system are given by the heavy solid lines and measurements in the 1-cm system by lighter lines.*

for the individual 200-s growth measurement periods of a run are connected by line segments and show both systematic and nonsystematic fluctuations. At very low density, the nonsystematic fluctuations were larger and decreased as growth proceeded. This can be seen by comparing several different runs, but was also evident from the calculation of errors given in the output. Typically, sd_λ for individual 200-s periods was ±0.06 to 0.1/h for the measurements at the lowest turbidity shown for both the 10-cm and 1-cm systems and became less than ±0.03/h for the 10-cm system and ±0.006 to 0.01/h for the 1-cm system when the turbidity became higher. The smallest sd_λ for the 1-cm runs shown in Fig. 2 was ±0.004 to 0.005/h, which in only slightly greater than the error attributable to the measurements. These very low errors were found only in association with regions where λ was quite constant. This suggests that the measured values of sd_λ included the effects of growth rate changes lasting more than a single measurement interval. We have attempted to estimate the average error not attributable to fluctuations lasting longer than one interval by averaging all the sd_λ data, over a range of cell densities, after eliminating any sd_λ datum where the previous or succeeding measurement of λ was more than 30% different. This does not bias the estimation of fluctuations due to photometric variation and to short-term fluctuations in growth rate, while it eliminates those whose period is longer than one period. By this criterion, average values of sd_λ for both of the two light paths progressively decrease from ±0.08 to ±0.03/h as density increases over the usable photometric range. Thus, under optimum conditions the error in the doubling time of a hypothetical culture that doubles every 30 min is ±0.6 min in a single 200-s measurement interval.

Based on these estimates of random error, three features of the nutrient broth growth curve emerged as systematic changes. First, there was a gradual slowing of growth rate. In the density range of 2 to 150 μg/ml, the rate dropped from about 2 to 1.5/h. Second, there was an abrupt drop above 150 μg/ml. Third, there was a temporary decrease; the specific growth rate passed through a minimum of less than half the normal rate when the culture reached 15 to 18 μg/ml. The slowing and recovery occupied 10 to 15 min.

The gradual slowing occurred in 4× concentrated nutrient broth, suggesting the buildup of toxic substances (data not shown). The abrupt drop above 150 μg/ml was due to nutritional factors and did not occur in the 4× concentrated medium, nor was it prevented by using pure O_2 for aeration (which increases the effectiveness of aeration fivefold).

Temporary slowing in growth occurred reproducibly and at nearly the same cell density, whether the measurements were performed with the 10-cm growth chanber or with the 1-cm apparatus, in which the apparent absorbances (turbidities) were approximately 10-fold different. We show (Fig. 2) four growth curves in long

light path and two in the 1-cm cell, but we have 10 other growth curves showing the growth-slowing phenomenon at the approximately the same culture density. The growth minimum was found to vary slightly from batch to batch of nutrient broth and with the duration of autoclaving. With one batch, the culture density at which the growth minimum occurred was shifted to higher densities almost exactly in proportion to the concentration of broth.

Figure 3 shows that a similar phenomenon occurred in another, richer complex medium (Luria broth). The temporary growth slow-down in Luria broth took place from 75 to 100 μg (dry weight)/ml. The gradual slowing was less pronounced. Neither effect was altered when O_2 was used instead of air.

The phenomenon of the temporary growth slow-down is shown here (Fig. 3) to be a nutritional one having to do with the nature and the level of the nutrients in the complex media. It is a diauxie comparable to the one found by Monod (6) in his original work with glucose plus lactose. In Monod's work, glucose is a preferred carbon source and the *lac* operon proteins, β-galactosidase and galactoside permease, needed for lactose utilization, are made only as the glucose is depleted. Many possibilities for nutritional diauxie are conceivable based on the components of the complex media. Conceivably, the block might be overcome either by utilization of something else in the medium, requiring a new transport system, or by the synthesis of the missing substance from other substances present, requiring a metabolic system. Nutrient broth medium contains 5 g of peptone and 3 g of beef extract per liter, whereas Luria broth (5) contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter. The point in the growth curve in nutrient broth where the growth slow-down occurred was shifted to the higher density characteristic of Luria broth by adding 5 g of yeast extract per liter. This addition also caused the culture to grow at the faster rate characteristic of Luria broth. The pause in growth was not eliminated by supplement of 19 L-amino acids or the components of a trace element mixture (7), by 10 μM $ZnCl_2$, or by 50 μg each of adenosine, uridine, and cytidine per ml. The pause was only partially relieved by lower levels of yeast extract or tryptone.

The growth diauxie was most prominent if the inoculum had been maintained for many doublings in nutrient broth at less than the critical density, and it was markedly reduced or obliterated if a growing but dense culture in nutrient broth was the source of the inoculum and dilution was such that only several-fold growth occurred before reaching the critical 15 to 20 μg (dry weight/ml in the fresh nutrient broth. The phenomenon of a temporary growth slow-down was not observed in dozens of trials in minimal M9 medium with glucose or succinate as the carbon source (data not shown) spanning densities of 1 to 200 μg/ml. No temporary growth slow-down occurred in cultures recently shifted up from glucose-M9 to nutrient broth (Fig. 4, curve A). However, it was observed after longer growth at low densities in nutrient broth (Fig. 4, curves B and C). Thus, to overcome the changes in the medium, the metabolic and/or transport system is quickly turned on, but is more slowly lost as growth ensues in a way that is typical of

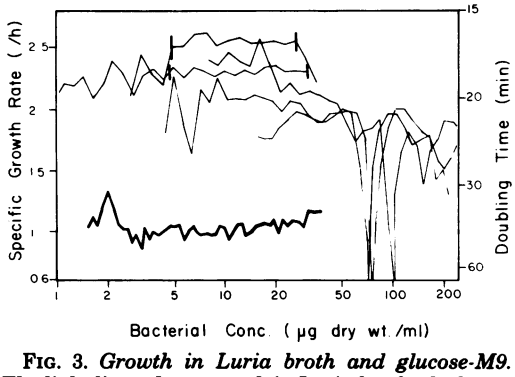

FIG. 3. *Growth in Luria broth and glucose-M9. The light lines show growth in Luria broth; the heavy lines show a 0.2% glucose-M9 culture. They are typical of many additional runs. The variation from one measurement of λ to the succeding measurement of λ is that expected from the observed sd_λ, except where the growth rate slows or accelerates. For the cultures initiated in the 10-cm apparatus a statistical analysis is shown in Table 1.*

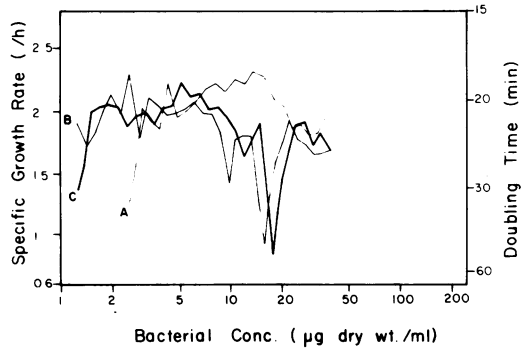

FIG. 4. *Growth in nutrient broth after shift-up from glucose-M9. (A) Growth rate of a culture immediately after shift-up. (B) Culture had been shifted up so that threefold growth occurred before the start recorded in the figure. The cells for the experiment of (C) had undergone 50-fold growth in nutrient broth before the start of the curve.*

regulatory control of the synthesis of proteins and not typical of that of mutation and selection.

Conditions where growth rate remains (very nearly) constant. One way to maintain environmental conditions constant is to keep the bacterial density low. It was for this reason that we had the 10-cm cell fabricated. But, as seen above, there was no region of density where growth was constant in nutrient broth. However, by two criteria, the portions of the growth curves in the region between the vertical bars shown in Fig. 3 in Luria broth and glucose-M9 for the lower density ranges were constant within the experimental error. The statistical data are shown in Table 1. For the Luria broth cultures, the standard deviation of the specific growth rate, sd_λ, was similar to the average of sd_λ values for the same set of successive measurement intervals. This comparison of the two estimates of the standard deviation is equivalent to an analysis of variance separating within-interval errors from between-interval errors. If there were an upward or downward trend in the specific growth rate, then the first estimate would be larger than the second. Even with no long-term trend, if there were fluctuations in the growth rate whose periods were longer than the interval of measurement, then the sd_λ would be greater than $\overline{sd}_\lambda$. The second criterion of constancy is that the slope of the linear regression of the specific growth rates versus the time that the interval began is less than its standard deviation. This condition was also satisfied by the two Luria broth cultures. The statistics for the third growth curve with Luria broth are shown in Table 1; there was a significant slowing (4% per h) in growth rate. A culture in minimal medium (0.2% glucose-M9) shown in Fig. 3 by the heavy line has also been statistically analyzed in Table 1. Its growth rate increased 1% per h but was less than the error of measurement. Fluctuations with longer terms than the 200 s are significant since, again, $sd_\lambda > \overline{sd}_\lambda$.

The data in this portion of the table discussed so far are based on about 31,000 measurements of turbidity. The remainder concern growth in 0.02% succinate-M9 and are based on more than 100,000 measurements. Because growth is slower in succinate, the measurements were made with 20-min intervals of data collection. In all cases in Table 1 the statistical treatment indicated that there was an upward trend in specific growth rate averaging 2.7% per h. Consistent with this was the finding that the standard deviation of the specific growth rate measurements was greater than the average of the sd_λ values.

An experiment to measure the long-term trend in growth rate in 0.02% succinate-M9 medium is shown in Table 2. A culture was grown in this medium and aerated at 37°C for 24 h after the succinate had been exhausted. Then

TABLE 2. *Growth of E. coli during repeated subculture in 0.02% succinate-M9 medium*

Run	No. of 20-min intervals in run	Time run started (h)	Specific growth rate (h^{-1})		
			$\bar{\lambda}$	sd_λ	$\overline{sd}_\lambda$
A	18	1.35	0.408	±0.1392	±0.0020
B	15	7.03	0.552	±0.0850	±0.0024
C	17	13.17	0.575	±0.0770	±0.0023
D	14	20.15	0.699	±0.0740	±0.0028
E	16	27.13	0.719	±0.0442	±0.0026
F	14	33.75	0.731	±0.0495	±0.0027
G	16	39.33	0.739	±0.0382	±0.0032
H	10	45.43	0.765	±0.0152	±0.0017
I	10	51.05	0.791	±0.0261	±0.0027

TABLE 1. *Statistical test for constant growth in the 10-cm system*

Medium	No. of intervals in run	Specific growth rate			Rate of change of λ[a] (slope [h^{-2}] ± SD)
		Avg rate $\bar{\lambda}$ (h^{-1})	Standard deviation		
			sd_λ[b]	$\overline{sd}_\lambda$[c]	
Luria broth	12[d]	2.54	±0.038	±0.042	−0.002 ± 0.055
Luria broth	20[e]	2.29	±0.057	±0.033	0.044 ± 0.035
Luria broth	21	2.36	±0.055	±0.036	−0.095 ± 0.026
Glucose-M9	51[f]	1.03	±0.079	±0.031	0.012 ± 0.013
Succinate-M9	11	0.61	±0.0270	±0.0036	0.015 ± 0.006
Succinate-M9	9	0.62	±0.0118	±0.0047	0.010 ± 0.003
Succinate-M9	20	0.54	±0.067	±0.0025	0.021 ± 0.006
Succinate-M9	17	0.60	±0.052	±0.0027	0.019 ± 0.006

[a] Slope of linear regression (and standard deviation [SD]) of λ versus time of start for the intervals in the run.
[b] Calculated as the standard deviation of all the individual specific growth rates of the intervals of the run.
[c] Calculated by averaging the standard deviations of the specific growth rate for all the intervals of the run.
[d] Treatment of the data delineated by the upper set of vertical bars in Fig. 3.
[e] Treatment of the data delineated by the lower set of vertical bars in Fig. 3.
[f] Treatment of the data from the entire run, shown by the heavy line in Fig. 3.

the culture was diluted 40-fold under sterile conditions into fresh medium to continue growth. It was diluted also by the same factor into the clean, but not sterile, 10-cm growth system. Two fresh subcultures were subsequently made in the same manner, when the growth of the culture in the spectrophotometer had reached a preset limit, through nine successive subculture passages. The statistical data are shown in Table 2 in a form similar to those of Table 1. Only specific growth rates for 20-min intervals were considered if sd_λ was less than 1% of λ; for most runs this meant that the first two or three 20-min intervals were eliminated from analysis. This was the case in run A; but growth did occur immediately during the first 20 min after the starved culture was diluted into fresh medium with a 355-min doubling time. The growth rate continued to accelerate throughout the entire observation period. The average specific growth rate increased progressively from run A, where the doubling time was 102 min, to run I, where the doubling time was 52 min. In all runs, the growth rate fluctuated more than would be predicted by the precision of the specific growth rate of 20-min intervals, and in most cases the change in growth rate in successive intervals was larger than its error, although the growth rate did not always increase during a run. But growth did not become steady even during this 60-h period, during which scrupulously constant conditions were maintained and where the total increase in biomass was in excess of 10^{14}-fold.

DISCUSSION

The apparatus described here has many potential applications in bacteriology and for cell culture generally. The aerating cuvette is the key part of the design; other double-beam spectrophotometers and other minicomputers could be used. We have reduced methodological error to the point that it is worthwhile to ask "How constant is bacterial growth during batch culture?" The answer is that the fluctuation in the specific growth rate, in the range of growth accessible with the 10-cm system for Luria broth and glucose-M9, is about the value projected from the data collected within individual 200-s intervals and shows no trend. The fluctuation in specific growth rate in these cultures when the apparatus is functioning optimally is about ±0.03/h. Therefore growth occurs with, at most, very small fluctuations. With ordinary nutrient broth, there are both a progressive slow-down and a temporary slow-down within the cell density range assessable with the 10-cm system. With the 1-cm cuvette system, progressive growth slow-down and abrupt growth slow-down, signaling the beginning of stationary phase, have been quantitated. These are phenomena familiar to bacteriologists, and the magnitude of the growth rate changes and the densities at which they take place depend on medium, oxygenation, etc.

Campbell (2) defines balanced growth as a process in which every extensive property increases with the same rate constant. Evidently the cultures must be kept below 2 to 5 μg/ml for nutrient broth and Luria broth cultures but can be allowed to reach considerably higher densities in glucose-M9. All the cultures used for this study came from inocula which, unless stated otherwise, were kept below those culture densities and may have actually been in balanced growth according to this definition.

Certainly the succinate-M9 cultures were not in balanced growth but exhibited long-term fluctuations over several-hour periods and a progressive increase in growth rate during the several days of subculture. These were not genetic changes, since the increase in growth rate was not inheritable. In addition the growth rate shifted only gradually; if it had been a genetic change, the growth rate would have remained fixed until the new faster variant had become an appreciable part of the population, at which point it would have changed abruptly. The data presented here document our impression gained over many years that the growth rate of *E. coli* in succinate minimal medium has a day-to-day variation that is large compared with that in other media and that continued subculture increases the growth rate, if growth is never allowed to stop.

The temporary growth slow-down seen with the complex media is a new phenomenon, but not an unexpected one. The purpose of this communication is not to characterize the detailed process of this growth slow-down, which most likely has to do with the utilization by the bacteria of certain preferred oligopeptides, purines, or pyrimidines. Rather it is to point out that bacterial cultures may have been subcultured in the same complex growth medium for extensive periods of time and harvested at identical densities, and yet not be physiologically the same. Physiological properties such as inducibility and degree of catabolite repression cannot be expected to be constant when there is a temporary slow-down. However, with ordinary methods for measuring the growth of cultures, workers have not, and probably could not, have been able to detect this growth slow-down phenomenon.

ACKNOWLEDGMENT

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