Control of Growth Rate by Initial Substrate Concentration at Values Below Maximum Rate

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The hyperbolic relationship between specific growth rate, $\mu$, and substrate concentration, proposed by Monod and used since as the basis for the theory of steady-state growth in continuous-flow systems, was tested experimentally in batch cultures. Use of a *Flavobacterium* sp. exhibiting a high saturation constant for growth in glucose minimal medium allowed direct measurement of growth rate and substrate concentration throughout the growth cycle in medium containing a rate-limiting initial concentration of glucose. Specific growth rates were also measured for a wide range of initial glucose concentrations. A plot of specific growth rate versus initial substrate concentration was found to fit the hyperbolic equation. However, the instantaneous relationship between specific growth rate and substrate concentration during growth, which is stated by the equation, was not observed. Well defined exponential growth phases were developed at initial substrate concentrations below that required for support of the maximum exponential growth rate and a constant doubling time was maintained until 50% of the substrate had been used. It is suggested that the external substrate concentration initially present "sets" the specific growth rate by establishing a steady-state internal concentration of substrate, possibly through control of the number of permeation sites.

The classic studies of Monod (11) on the growth of bacteria resulted in the formulation of an equation expressing the relationship between specific growth rate and substrate concentration, when substrate is the limiting nutrient, as shown below:

$$\mu = \frac{(\mu_{\text{max}}S)}{(K_s + S)}$$  (1)

In this equation $\mu$ is the growth rate constant, or specific growth rate, and $\mu_{\text{max}}$ is the maximum growth rate constant for the organism, substrate, and cultural conditions specified. The saturation constant, $K_s$, is also characteristic of a specific system (organism, substrate, and cultural conditions) and is defined as the substrate concentration at which $\mu = 0.5 \mu_{\text{max}}$. The symbol $S$ has been used as initial substrate concentration, $S_0$, in subsequent studies (14, 17) of other organisms and limiting factors, although it is clear from the original work that the symbol was used to represent $S_t$, the substrate concentration at the time of measurement of $\mu$. If $\mu$ is calculated from the initial rate of growth, then $S_0$ and $S_t$ are essentially equivalent unless a significant proportion of the initial substrate is consumed during a lag period before a measurement of $\mu$ can be made.

It was the latter consideration which prompted Monod's choice of a method for estimating the value of $K_s$ (11). Measurements of $\mu$ for a range of initial substrate concentrations showed that $\mu$ was decreased below $\mu_{\text{max}}$ only at very low initial substrate concentrations for the systems he investigated. These data indicated that $\mu$ was controlled by $S$ and that the concentrations at which significant variations in $\mu$ could be expected to occur were so low that a significant error would be introduced by using $S_0$ as the value of $S$ in the equation. Having shown that $Y$, the bacterial mass produced per unit of substrate consumed, was constant throughout the growth cycle and that growth ceased at the point of substrate exhaustion, Monod made the assumption implicit in equation (1) that $\mu$ is tightly coupled to $S$, i.e., that a change in $S$ during growth of a culture results in a corresponding change in $\mu$. He then measured optical density of a number of cultures during growth on known amounts of initial substrate and calculated, for small intervals
throughout the growth cycle, the substrate consumed (from $Y$ and the change in optical density), the average substrate concentration present during the interval, and the average $\mu$ during the interval. A plot of these calculated values for $\mu$ and $S$ resulted in the familiar hyperbolic curve described by equation (1) and allowed an admittedly imprecise estimate of the value of $K_s$. For *Escherichia coli* in minimal medium in which the carbon source was the growth-limiting nutrient, values of $K_s$ were estimated as 4, 2, and 20 mg/liter for growth on glucose, mannitol, and lactose, respectively. Values of $K_s$ were not determined for *Bacillus subtilis*, but measurement of $\mu$ for a range of initial substrate concentrations indicated that $K_s$ would be very small since growth rate was affected only at very low concentrations. It, therefore, seemed likely that very small values of $K_s$ could be expected as a general rule in bacterial growth.

Monod tested the validity of equation (1) by using it to calculate growth curves based upon the values determined for the growth constants. (It was necessary to choose the time origin to allow for the lag phase which the equation cannot predict.) By using a single set of values for $K_s$ and $\mu_{\text{max}}$, determined as described above, it was possible to plot calculated curves which exactly coincided with experimental growth curves for *E. coli* on initial glucose concentrations ranging from 200 to 25 mg/liter and on lactose at 180 to 60 mg/liter.

Later determinations of the value of $K_s$ were made by Schaefer (17) for *Mycobacterium tuberculosis* growing on glucose as the limiting nutrient in a complex medium and by Novick and Szilard (14) for a tryptophan auxotroph of *E. coli* in lactate medium with tryptophan as the limiting nutrient. In both cases, only initial concentrations of the limiting nutrient were considered and hyperbolic plots were obtained for $\mu$ versus $S_0$. Novick and Szilard stated that growth rates "must be taken from the early part of the growth curve" because the tryptophan concentration would decrease during growth. No experimental data were presented to support the assumption that a change in external concentration of tryptophan would lead to an immediate corresponding change in growth rate, and growth data from which $\mu$ values were calculated were not shown. It is therefore not possible to determine whether exponential growth was observed at growth rate-limiting concentrations of tryptophan, i.e., whether doubling time was constant during a period when $S$ was changing. However, semilogarithmic plots of data from which $\mu$ values were obtained were presented by both Monod (11) and Schaefer (17). In both studies, exponential growth, i.e., growth at a constant value of $\mu$, was observed at initial substrate concentrations considerably below those required for growth at the maximum rate. This would clearly be impossible if equation (1) is an exact statement of the controlling influence of $S$ upon $\mu$. Indeed, Herbert et al. (6) have stated that $\mu$ and $t_d$ (doubling time) are constants "only when all substrates necessary for growth are present in excess." This is an assumption which has never, to our knowledge, been subjected to experimental verification, and one which is apparently inconsistent with the data of Monod and Schaefer cited above, since substrate concentration (which was not measured in either study) must have changed during the period when $\mu$ remained constant. In Schaefer's study, for example, doubling time (or $\mu$) was constant for 120 hr in cultures at an initial glucose concentration as low as 5 mg/ml, although $K_s$ for this system was 4.5 mg/ml.

Two related assumptions have thus been widely accepted without complete experimental proof. The first is the assumption that equation (1) represents an instantaneous equality, i.e., that a change in $S$ during growth results in an immediate change in $\mu$ if $S$ is at a level below which allows $\mu_{\text{max}}$. The second, which is a corollary of the first, is that stated by Herbert et al. (6) that exponential growth ($\mu$ and $t_d$ constant) can occur in a batch culture only when $\mu = \mu_{\text{max}}$. The use of equation (1) in development of the theory of continuous culture (6, 12) was based upon the assumption of an instantaneous response of $\mu$ to a small change in $S$ (substrate concentration in the reactor) as the mechanism by which the steady state in $S$ is maintained. Whereas the original theory was concerned with prediction of response to small changes, the equations were developed as a dynamic model, not solely as a description of a series of equilibria as Young et al. (20) have recently argued, since equation (1) would theoretically depict $\mu$ during small transients. A number of recent investigations concerned with the kinetics of continuous cultures subjected to sudden large changes in dilution rate (1, 4, 5, 10) or concentration of limiting nutrient in the feed (13, 18) have shown that response of $\mu$ is delayed, i.e., that the Monod equation and the equations for continuous culture based upon it are not adequate for prediction of transient behavior during periods when a chemostat operates with changing substrate concentration (i.e., somewhat as a batch culture although with continuous replacement of culture by fresh medium).

In view of this inadequacy of the continuous-
flow equations during the transient state, it seems important to test the validity of the initial assumptions. In the systems studied by Monod, the extremely small value of $K_s$ did not allow direct experimental verification of the relationship between $\mu$ and $S$ since the substrate concentrations of critical interest were too small for precise measurement. The prediction of an experimental growth curve by using constants obtained by calculations which were essentially the reverse of those used for prediction was not a completely satisfactory test of the relationship. Only actual measurement of $\mu$ and $S$ throughout the batch growth cycle would constitute a valid test of equation (1), and this would require use of a system with a $K_s$ sufficiently high to allow measurement of $S$ in the range of critical interest, i.e., when $S$ is equal to and less than $K_s$.

In many investigations of the growth of mixed microbial populations in our laboratory (3, 15, 16), it has been found that $K_s$ values are often 10 to 20 times greater for these systems than for those studied by Monod. It has also been found that extended periods of exponential growth occur in batch systems at growth rate-limiting initial concentrations of substrate and that plots of $\mu$ versus $S_0$ yield a hyperbolic curve and values of $\mu_{\text{max}}$ and $K_s$ which rather adequately predict equilibrium levels of cell and substrate concentrations in continuous flow systems. The use of $S_0$ as the value of $S$ in equation (1) is consistent with the methodology originally described by Monod for evaluation of $K_s$ in systems wherein $K_s$ is sufficiently large to allow limitation of growth rate at concentrations of substrate which will not be significantly decreased during a lag period (cf. reference 17). However, maintenance of a constant doubling time (exponential growth) at these substrate concentrations for an extended period of time is inconsistent with the assumed instantaneous control of $\mu$ and $S$ with the related assumption that exponential growth occurs only when all components of the medium are present in excess. This apparent contradiction of the hypothesis upon which continuous culture theory is based indicates that the observed deviations from predicted behavior during the transient state (18) may be simply another manifestation of the deviations we have observed in batch systems. The investigation reported herein and others reported elsewhere (A. F. Gaučy, Jr., et al., submitted for publication) were undertaken to determine the extent to which growth rate fails to respond to changes in $S$ during batch growth. For this purpose, it was necessary to isolate an organism capable of growing on minimal medium with a high $K_s$ and to measure both growth and substrate concentration throughout the growth cycle.

**MATERIALS AND METHODS**

Several microorganisms were isolated from municipal sewage and screened for the range of dependence of $\mu$ on substrate concentration in glucose minimal medium. A microorganism exhibiting a rather wide spread of $\mu$ values for high and low initial substrate concentrations was selected for further study, since such an organism would be expected to exhibit a $K_s$ value sufficiently large to allow adequate assessment of substrate removal during the course of exponential growth at a substrate concentration for which $\mu$ was dependent on $S$. During the investigation, the organism was maintained on nutrient agar (Difco) slants and an inoculum from a fresh slant was grown through two transfers on glucose minimal medium (see below) prior to use in a growth experiment. Frequent streak plates were made as a check on the purity of the culture.

The organism was identified as *Flavobacterium* sp. on the basis of the following characteristics. It is a small, nonmotile, gram-negative rod, aerobic and heterotrophic. Growth on nutrient agar is abundant. Colonies are raised and shiny with a brownish yellow, nonsoluble pigment becoming yellow-orange in the center. On glucose, lactose, and sucrose broth, there is very slight production of acid after 10 to 20 days; no gas is produced. No acid or gas is produced in broth containing mannitol. Litmus is reduced within 7 days. Starch, gelatin, and tributylin are hydrolyzed. There is no growth on sodium citrate (Koser's medium) or on nutrient agar containing 7% NaCl. The organism is urease-negative and does not fix $N_2$, reduce nitrate, or produce indole.

The basal salts medium used for growth studies contained the following components (amounts per liter): $(NH_4)_2SO_4$, 500 mg; MgSO$_4$·7H$_2$O, 100 mg; MnSO$_4$·H$_2$O, 10 mg; CaCl$_2$, 7.5 mg; FeCl$_3$, 0.5 mg; phosphate buffer (1.0 m, pH 7.0), 10 ml; tap water, 100 ml; distilled water to volume. Glucose solution was sterilized separately and added to sterile medium to the desired concentration. Cells were grown in Erlenmeyer flasks fitted with 18-mm diameter test tubes to allow frequent determinations of optical density. Aeration was accomplished by growth on a reciprocal shaker at 120 oscillations/min. Temperature was 27 $\pm$ 1 C.

Growth was measured as optical density at 540 nm by using a Bausch & Lomb Spectronic 20 colorimeter. A correlation curve for dry weight of cells versus optical density is shown in Fig. 1. Dry weight was determined by drying to constant weight cells harvested from measured volumes of culture by filtration through predried and weighed membrane filters (0.45-nm pore size).

For the experiment in which utilization of carbon source was measured, a series of replicate flasks was prepared. One flask was removed at each sampling time for determination of dry weight of cells and of substrate remaining in the medium (using the filtrate from the dry weight determination). Growth was fol-
Fig. 1. Correlation curve for optical density, measured at 540 nm, with biological solids (dry weight of cells, mg/liter).

The weaver-Burk plot the value of the optical density the following equation

\[ \log \text{COD} = \text{initial mg/l} + t \cdot \mu \]

where \( t \) is time in hours. The initial COD value is the intercept on the y-axis. The slope \( \mu \) is related to the rate constant by

\[ \mu = 0.693/t_d \]

(2)

The symbol \( t_d \) designates the time for optical density (or bacterial mass) to double, and the numerator is the value \( \ln 2 \). Numerical values for \( \mu_{max} \) and \( K_s \) were obtained from the data by using a straight line form of equation (1), i.e., from a double reciprocal (Lineweaver-Burk) plot similar to those employed in studies of enzyme kinetics.

\[ 1/\mu = (1/\mu_{max}) + [K_s/(\mu_{max} \cdot S)] \]

(3)

RESULTS

Figure 2 shows the course of substrate removal and growth for a system in which the initial concentration of carbon source was 340 mg/l COD (320 mg/l glucose). From the semilogarithmic plot it may be discerned that the culture grew with a constant doubling time for a period encompassing the 2nd and 6th hr. Thus, the exponential growth phase terminated at approximately the 6th hr, and during this time a significant amount of carbon source was removed, approximately 175 mg/l COD, or half of the exogenous carbon source originally present. Thus, it is apparent that, during the experiment, \( S \) changed considerably during the period of constant doubling time, i.e., during the period for which the growth could be characterized as an exponential growth phase with a numerically definable value for the growth rate constant, \( \mu \).

The fact that nearly 50% of the carbon source was removed during the period of exponential growth would not be a particularly significant finding if the initial concentration of carbon source had been above that required to permit generation of the maximum exponential growth
rate, i.e., if the initial concentration of substrate had been in excess. However, the value of \( \mu = 0.315 \text{ per hr} \) was sufficiently below the \( \mu_{\text{max}} \) value for the system to adjudge it to be in the substrate concentration-dependent range. This can be seen from Fig. 3, which shows plots of \( \mu \) versus \( S_0 \) and \( 1/\mu \) versus \( 1/S_0 \) for five experiments in which growth was measured for cultures at various initial substrate concentrations. The value of \( \mu \) from the data of Fig. 2 is designated as experiment 5 in Fig. 3. At all substrate concentrations shown, well defined exponential phases of growth were observed. In several of these experiments covering a range of initial substrate concentrations, a sample was removed for substrate analysis (COD) near the end of the exponential phase of growth. In all cases, significant percentages of substrate removal, consistent with that shown in Fig. 2, were observed. The values of \( \mu_{\text{max}} \) (0.38 per hr) and \( K_s \) (100 mg/liter) from the straight line plot of Fig. 3 were substituted into equation (1). The hyperbolic curve calculated from this equation is shown in the figure, and it can be seen that it does provide a reasonable representation of the data.

**DISCUSSION**

It is apparent from the data shown herein that equation (1) does not accurately describe the relationship between \( \mu \) and \( S \) during growth of a batch culture and therefore probably should not be expected to predict behavior of a continuous culture during a severe transient. It is also apparent that the two methods described by Monod (11) for estimation of \( K_s \) do not result in identical values for that constant. Unless \( \mu \) responds instantaneously to changes in \( S \), a plot of \( \mu_t \) versus \( S_t \) for intervals throughout the growth cycle would be quite different from a plot of \( \mu \) versus \( S_0 \) (as in Fig. 3), wherein each value of \( \mu \) represents the exponential growth rate constant for one culture. In other words, equal concentrations of substrate, present as \( S_0 \) and \( S_t \), do not generate equal specific growth rates. The magnitude of the difference in values of \( K_s \) obtained by the two methods would depend upon the magnitude of the delay in response, or the degree of "looseness of coupling" of \( \mu \) to \( S \). If the optical density data shown in Fig. 2 were used to calculate \( \mu \) and \( S \), as Monod did, and if \( \mu \) were plotted versus the calculated values of \( S \), one would obtain a much smaller value (approximately 25 mg/liter) for \( K_s \) (a hyperbolic plot of much steeper curvature).

Since \( K_s \) is an important constant in equations predicting levels of cells and substrate in continuous flow systems, it now becomes important to consider whether \( S_0 \) or \( S_t \) is more properly used for its evaluation in batch systems. Although new equations must undoubtedly be developed to describe the transient state in continuous cultures subjected to severe disruptions, the long experience of many investigators with maintenance of the steady state in such cultures indicates that the present equations are adequate for description of operation at steady state or with only small disturbances due to variation in \( D \) (dilution rate) or in-flowing substrate concentration. Determination of \( K_s \) in the continuous flow system by setting \( D \) equal to 0.5 \( \mu_{\text{max}} \) and determining \( S \) was proposed by Herbert et al. (6) as the most accurate and easily achieved method, and \( K_s \) values have commonly been determined in this way or by calculation from one of the steady state equations. However, validation of the theory would seem to require correlation of actual measurements of \( K_s \) under both batch and continuous flow conditions.
The "slippage" in the Monod equation demonstrated herein and in similar studies with other organisms (Gaudy et al., submitted for publication) indicates that the relationship between \( \mu \) and \( S_0 \) may have significance not previously appreciated, which may be applicable also to continuous flow systems. These data, in conjunction with other work cited below, suggest the following working hypothesis to explain the control exerted by \( S_0 \) (not \( S_t \)) on the exponential growth rate constant, \( \mu \). In batch systems, the specific growth rate, \( \mu \), as shown herein, is determined by the concentration of external substrate initially present. The exponential rate of growth, once established, is not readily changed by changing external concentrations of the growth-limiting nutrient in either batch or continuous flow systems. Thus, the culture exhibits a considerable inertia, or resistance to change in rates of synthetic processes, which must be attributed to the action of one or more metabolite controls. Since bacteria are generally capable of concentrating most nutrients against a considerable gradient, it is difficult to visualize an inertial control that is not based upon control of the rate of accumulation of the limiting nutrient. It seems apparent that during the lag phase in a batch culture, whether long or short, the growth rate is "set" and that the rate is determined by the external concentration of limiting nutrient, most probably by control of the amount of synthesis of proteins involved in uptake. In this connection, several recent studies of uptake and growth are of particular interest. Kubitschek has shown that uptake is constant throughout most of the cell cycle (interval between divisions) for Escherichia coli (8) and that the growth of the cell is linear (9). He has suggested that "the constancy of cell growth depends on the presence of a growth-limiting constant number of active sites (for uptake), which does not change until late in the cycle" (9). Winkler (19) has recently demonstrated that for carrier-mediated transport a steady state is established in which efflux operates to maintain a constant intracellular level of substrate. Koch and Coffman (7) have shown that, upon induction of uninduced cells, entry of the substrate becomes rate-limiting.

These data, along with those presented herein, suggest that the resistance to change in growth rate in a batch culture, and possibly in a continuous flow culture as well, is explained by control of the number of "permeation sites" by the concentration of external substrate to which the cell is exposed during the period when adjustment of the rates of all synthetic processes for achievement of balanced and orderly growth is occurring, i.e., during the lag phase in a batch culture or during steady-state growth in a continuous culture. The cell thus establishes a steady-state internal concentration of the growth-limiting nutrient, and the grow rate thus established can be maintained despite a significant decrease in external concentration of that nutrient. The response of \( \mu \) to the decrease in \( S \) during growth of a batch culture may then occur (i.e., exponential growth may cease) only when the substrate concentration in the medium, \( S_t \), becomes too low to allow maintenance of the established steady-state internal concentration. If this hypothesis is correct, \( K \) may be related in some manner to the saturation constant for the transport system or binding protein involved in uptake, and the similarity between the Monod equation and the Michaelis-Menten equation may be less than fortuitous despite dissimilarity of the kinetic systems (Gaudy et al., submitted for publication).

The observations and conclusions reported herein are based solely upon studies of batch cultures in which initial substrate concentration is rate-limiting. Much additional study of both batch and continuous flow systems is needed before the relationship between \( \mu \) and external substrate concentration can be fully understood. However, it is possible to conclude at present that the Monod equation does not accurately describe this relationship during growth of a bacterial culture in batch and therefore must also be expected to be inadequate for the description of transients in continuous flow.

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