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Microbial Physiology and Ecology of Slow Growth

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INTRODUCTION

Under conditions where all the components needed for energy transduction and formation of cell constituents are supplied, bacteria can grow and adapt to the ambient milieu. Microbial physiologists, by avoiding consideration of lag phases and stationary phases, have learned a lot about cellular processes by studying cells during exponential balanced growth, i.e., after the lag phase and while the growth rate remains constant. The steady-state growth rate, depending on the medium, may be quite slow or quite fast, but there are limits to either extreme. Under poor conditions, instead of growing more slowly the cells may form resistant forms, such as spores, or periodically enter and leave other quiescent states. Under very favorable conditions, the cells may grow quite fast, but they cannot grow infinitely fast because the growth rate is ultimately limited by cellular processes and not by the medium constituents. Consequently, the growth rate does not increase indefinitely as individual components of the medium are augmented (46). Fast growth is important but is of only indirect interest here, since this review deals with the problems that free-living saprophytic unicellular organisms face when placed in an adequate but poor environment.

There are many kinds of growth states. The "gold standard" and the simplest is the theoretical and unnatural case of balanced growth under the constant, invariant, conditions of a chemostat or turbidostat. While only temporarily supporting a nearly constant growth rate, ordinary batch growth from a small inoculum approximates growth in the turbidostat, but only after the lag phase has passed and before growth slows because of depletion of resources or pollution resulting from overcrowding of the organisms. This state of balanced growth is the only one that lends itself to ready analysis, and this is the reason why microbial physiologists have studied it so diligently. Other more realistic and relevant growth states include different kinds of fluctuations in the environment and all of the actual problems faced by organisms in the real world, as well as contingencies generated throughout evolutionary history. These evolved processes have led to the many additional genetic facets and elaborations of the cell's biology beyond those needed to handle the idealized eternal steady-state situation that is characteristic of the continuous culture (53, 55).

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This review first considers balanced growth under sufficient but poor to moderate nutritional conditions such that the organisms do not sporulate, etc., but do grow and reproduce continuously. Then some variations on the theme are addressed.

CENTRAL PROBLEM OF STEADY-STATE UPTAKE COUPLED TO GROWTH

To grow continuously, the cell needs a constant supply of all the resources that are convertible into all of the constituents needed for cell propagation. This requires having uptake systems, mechanisms for intermediary metabolism, mechanisms for macromolecular metabolism, and mechanisms for cell division. Also needed are specialized mechanisms for degradation of certain macromolecules and the excretion of waste materials. I will include here all of the items that are important to the long-term but not the short-term ecology of the organism; these include secretion of protein, turnover of protein, cell death, and formation of storage materials. Each of these items is worth a review of its own. The global chemical reactions can be diagrammed in the broadest sense as follows:

$$\text{Uptake} \rightarrow \text{growth} \rightarrow \text{waste, etc.} \quad (1)$$

Extracellular processing and uptake in the gram-negative bacteria can include (i) degradation of a substance in the medium by enzymes secreted by the cell to convert certain macromolecules to utilizable forms; (ii) diffusion of the resource into the cell from the environment; (iii) movement of the cell to the resource; (iv) passage of substrates through porins; (v) transit of substrates through the periplasmic space; (vi) hydrolyses in the periplasmic space; (vii) attachment to binding proteins in the periplasm; (viii) uptake of substrates through the cytoplasmic membrane via permeases or other transport systems; (ix) release of the changed or unchanged molecule into the cytoplasm; and (x) conversion of a substrate as part of the transport event into a derivative of the original compound. Some of the possibilities in this list are alternatives. The list for gram-positive organisms would not include items (iv), (v), and (vi), although (vi) might be included by those who view the contents of the gram-positive thick wall as a "periplasm."

The list for the subsequent internal physiological phase includes (i) reworking of the input molecules into all the needed moieties for macromolecules and other polymers; (ii) transduction of potential free energy from reactants by facilitating chemical reactions that can occur spontaneously (exergonically) to produce chemical forms that can be used to drive cellular reactions (the alternative is transduction of light energy into a usable form); and (iii) assembly of functional macromolecules, by templated and nontemplated mechanisms, through the dehydration of the low-molecular-weight moieties generated by intermediary metabolism. (Degradation and excretion of wastes are not addressed in this review.) The salient point is that if all the individual steps (both chemical and physical) for uptake, growth, and excretion were counted, there would be many thousands of steps even for the simplest free-living cell.

The question is, "What limits growth under relatively poor growth conditions?" If we know which externally supplied single substance limits growth, we could add more of it until further addition has no effect. But the question then becomes, "What limits growth?" It is not always the uptake process. In many cases, the transport capability of the cell exceeds its growth capability. The reason for this growth limit ceiling may be a physical limitation, a biochemical limitation, or a design consideration that is an evolutionary "choice" of the cell that "tunes" it to act effectively within its biological niche.

When too much uptake occurs in a well-regulated cell, the growth machinery must affect the uptake systems instead of the reverse situation. This control of the transport systems is the key issue covered in this review. It is a significant point that has been underemphasized in the literature and has also been left out of the above two lists of external and internal processes. It was left out because it fits between the lists. I will review approaches that deal with steady-state metabolic systems and the coupling of uptake and growth, and then I will try to synthesize ideas and illustrate them by the use of a flexible computer simulation.

Previous System Approaches to Steady-State Metabolism

The study of kinetics of metabolic systems of enzymatic reaction steps has a long history starting from the time of Christiansen (17a, 17b). Workers in the field have included Hearon (33, 34), Savageau (97), and Kacser and Burns (38). More recently, Dean et al. (21), Dykhuisen (24, 25), Westerhoff et al. (111, 112), Kell et al. (42, 43), van Dam and Jansen (106), and Button et al. (8–13) have published in this area. Perhaps the best-known model is the metabolic control analysis (MCA) model of Kacser and Burns (38). This model has dominated the field since 1973. As in approaches before this time, equations for a series of enzymatic steps are linked by the assumptions that the rate of successive steps is constant and that the system is in a steady state. This means that the equations for the rate of the individual steps can be set equal to each other. For a series of reversible reactions, this leads to an insoluble system of equations. The system can be solved, however, by assuming that all the forward and backward steps are in their first-order region, and therefore the denominators in the equations for the individual steps can be neglected. Thus, for the simple reversible Briggs-Haldane (5) version of the development of the Michaelis-Menten enzyme kinetics (70), we could write, for the ith reaction:

$$v_i = (V_{\max i}/K_i)[S_i - (S_{i+1}/K_{-i})]/[1 + (S_i/K_i) + (S_{i+1}/K_{-i})] \quad (2)$$

where v_i is the velocity of the ith step, $V_{\max i}$ is the maximum velocity in the forward direction, S_i is the substrate concentration, and S_{i+1} is the substrate for the next enzyme and, of course, also the product of the ith step. K_{-i} is the Michaelis-Menten constant in the reverse direction. (Equation 2 is repeated in the computer terminology of Table 1, but there S_i is designated SI.)

S_{i+1} influences the reaction rate by its presence in both the numerator and denominator of equation 2. The importance of the term S_{i+1}/K_{-i} in the numerator is that it tends to reverse the uptake dependent on the effective overall equilibrium constant for the enzymatic or uptake process. The term in the denominator is probably crucial because it is there that product inhibition acts to influence the reaction rate. Product inhibition can act to control the reaction even if uptake is essentially unidirectional. For the MCA formulation, the denominators for each step are discarded for simplicity and solvability. Therefore, in the MCA formulation, instead of equation 2, the following is used:

$$v_i = (V_{\max i}/K_i)[S_i - (S_{i+1}/K_{-i})] \quad (3)$$

Although a system of such equations can be solved, it deletes a very important part of cell physiology; this part is retained in the scheme in Fig. 1. On the other hand, the scheme in Fig. 1

(see below) is impossible to solve analytically. Consequently, computer simulation is all that is available.

Mathematical Analyses of Uptake and Initial Phase of Intermediate Metabolism

The literature contains a number of theoretical attempts to analyze just the initial steps bringing substrates into the metabolism of the cell. The first and simplest approach was that of Monod (60–62, 71, 72). Monod assumed that uptake was hyperbolically dependent on the substrate, with a mechanism similar to a simple irreversible enzymatic reaction, and that this enzymatic step alone controlled cell growth. The uptake is carried out by the equivalent of an enzyme, a protein functioning to transport substances through the cytoplasmic membrane; Monod called it the permease (94). Although this model is a tremendous oversimplification, it has been the mainstay of both microbial physiology and ecology, and many thousands of papers have been written on the basis of this tacit assumption.

The first instance of a two-stage uptake formulation of which I am aware is that of Blackman (4). In a nonmathematical way, his 1905 model simply assumed that at low substrate concentrations the rate of uptake was proportional to substrate concentration and at high substrate concentrations both uptake and growth rate were independent of the substrate concentration because some other nutrient or intracellular (or intraorganismal) factor was now limiting. Powell (90) imagined that entry to the cell was governed by Fick's diffusion equation. For the present purposes, Fick's diffusion law is the same as that for a reversible enzyme reaction in which the V_{max} and K_m values for both the forward and back reactions are each very large. Powell also imagined that this first step was followed by an irreversible enzymatic mechanism for entry into the metabolic machinery of the cell. As I have discussed several times (51–53, 58, 60), this was preceded logically by Best's (3) derivation and followed by the independent derivations by Koch and Coffman (58) and by Dabes et al. (20). The four treatments are really the same and lead to the same equation. Thus, for this Best case, where a diffusion step precedes an enzymatic (hyperbolic) step, the solution is a quadratic one:

$$v = V_{max}(S + K_m + J)\{1 - [1 - 4SJ/(S + K_m + J)^2]^{1/2}\}/2J \quad (4)$$

The extra parameter is J, which is defined as $V_{max}/(AP)$ where A is the surface area of the bacteria and P is the permeability constant, i.e., the diffusion constant during passage through the membrane divided by the thickness of the membrane. As J, the third parameter of the equation, varies from zero to infinity, this equation fades from the hyperbola of Monod (71, 72) to the two straight-line segments of Blackman (4).

The next most elaborate treatment of which I am aware that generates an analytic solution is that of Koch (see equation 4 of reference 48) for reversible uptake followed by irreversible enzymatic utilization. An even more sophisticated model was formulated and solved (see equation 6 of reference 48); this further complicates the uptake process. Moreover, it is not appropriate for either of these to be considered further because both assume that once transport has been accomplished, cell consumption is fast.

The three parameters for the Best case, V, K, and J, are few enough that the results can be fitted usefully to sets of data. This model gives an accurate accounting of the substrate dependence of the growth of actual cultures and is more appropriate than the Monod model. The optimum formulation in this simplest case has a feature that allows growth to be limited by a step other than uptake steps.

One more approach to this subject in the literature is that of Button (11, 12); he designated his formulation Janusian because it has a stage that looks forward as well as one that looks back. Looking back to the external concentration, the rate is governed by productive collisions with porins and permease sites, which is given by collision frequency theory as a second-order rate constant, which Button designates specific affinity. With everything else being constant, at low substrate concentration, an organism with twice the specific affinity will grow at twice the rate, while at higher concentrations, the proportionality no longer occurs due to saturation of the sites in accord with Monod's type of thinking. Looking forward, the rate comes into equilibrium depending on all the resistances to flow prior to the final uses of the substrate. This relationship between driving force and flow of the various steps is collected into a translation coefficient, and if its value is known, a global formulation of the kinetic constants for the rate in the multicomponent system is possible. A steady-state solution of growth rate as a function of collision frequency, permease saturation, and downstream resistance is then available. In some sense, the formulation presented above expands on Button's ideas but with the important addition of mechanisms to link the two heads of the Roman god of doorways, Janus.

MODELING THE COUPLING OF UPTAKE CAPABILITY WITH GROWTH CAPABILITY

In this section, I will formulate a kinetic model for the uptake and utilization of a solute that illustrates the kinds of possible interactions for control of uptake by the ability of the cell to grow. To do this, I will formulate a general kinetic model. The model glosses over some aspects and combines others, but it emphasizes the mechanisms that link the two global processes of uptake and intracellular metabolism. It does not consider protein turnover or cell death. Nor does it consider the generation and limitation of energy sources, whether they be the proton motive force or be derived from high-energy phosphate bonds. I will commit these crimes because they are minor compared with the important aspect of the cell's control of its own transport, which was not adequately taken into account in earlier treatments.

The model (see Fig. 1) includes four possible ways for the limitations of the capability for growth to affect the concentration of an intermediate that is common to both the uptake and growth process. In the model, this intermediate is designated SI (for substrate inside the cell). When its concentration becomes too great, it regulates (limits or inhibits or diverts) the net import of substrate into the cell. It is assumed that a specific membrane-bound component, the transporter, T, can face the outside and the inside of the membranes and can be free or bind the substrate on either side of the membrane. The first of the four regulatory mechanisms is reversal of the uptake process via the rate constants $K8$, $K6$, $K4$, and $K2$. The second possibility is product inhibition of the transport process by binding the transporter on the inside of the cell; this is the factor omitted in the MCA treatment. The third possible mechanism is that the cell has ways to regulate transport activity by phosphorylation of T or control of an allosteric enzyme, etc. Alternatively, there may be a depletion of the cell's energy reserves for transport that limits growth.

The fourth possible regulatory mechanism is overflow metabolism. Overflow metabolism, also called spillover metabolism, is the diversion of some of the resource that had been pumped into the cell and funneled into its metabolism but then was converted into a substance that is excreted. In this seemingly perverse mechanism, the chemical flow leads to the con-

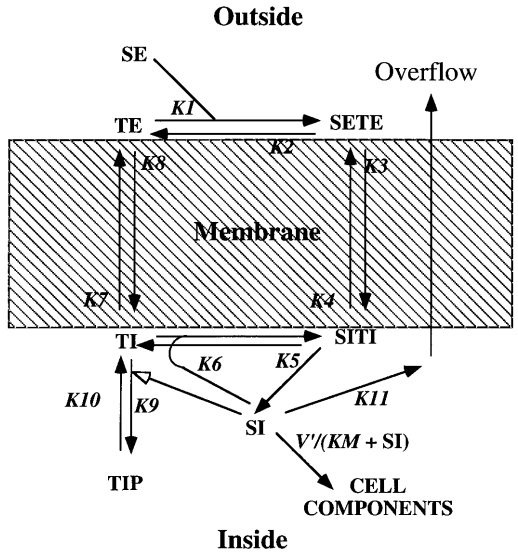

FIG. 1. Metabolic scheme for the linkage of uptake and growth. This is an inclusive scheme that includes the assumed junction point of the uptake and metabolism parts of the cell physiology, SI. The scheme was used to formulate the computer statements in Table 1 that would model the several control processes. See the text for details.

struction of compounds that are excreted "wastefully" from the cell. Classical cases are uptake and incomplete utilization of glucose, followed by the excretion under different conditions of either acetate, α-ketoglutaric acid, or glutamic acid by different organisms (53, 76, 78, 104). Because of this apparent wastage, only some of the glucose carbons are actually utilized for growth. This overflow metabolism is a different case from when glucose is fermentatively converted into cell materials and the waste products, ethanol plus CO_2, for example, are excreted. In the fermentation case, ethanol and CO_2 are stoichiometric waste products and their formation is not elective. However, there are several alternative excretory products, but their excretion serves the purpose of permitting the conservation of reducing equivalents. In the overflow case, the process is elective in the sense that the organisms could have metabolized the overflow substance completely with the available oxygen but did not "choose" to do so and instead processed additional glucose molecules. I would view the Crabtree effect, in which cells engage in fermentation in the presence of oxygen, as another example of overflow metabolism.

Please recognize that I am going very far out on a limb, because there is no evidence for any of these four regulatory processes. Transport systems have been extensively studied but usually only when decoupled from growth. Only a few studies have tried to investigate the regulation of the process in a way that is meaningful from the viewpoint of the physiology of the growing cell.

The metabolic scheme that is sufficient to couple transport and growth is shown in Fig. 1 and is represented in Table 1 in the corresponding kinetic system of equations written in computer language. The formulation is actually very spartan, even though it has features not previously presented in the literature. For example, it omits processes such as diffusion to the cell, diffusion through porins, and diffusion through the periplasmic space. These have been glossed over because the diffusion processes are usually (but not always [e.g., for diffusion of maltose oligosaccharides]) too rapid to be controlling or limiting elements in cell growth. In Table 1 and Fig. 1, capital letters are used to identify the components and kinetic parameters throughout to make the task of formulating and understanding the computer simulation easier. In this scheme, the external substrate (SE) reacts at the cell surface with the external form of the cell-bound transporter (TE). The product complex (SETE) moves (diffuses or undergoes a conformational change) to the inner face of the cytoplasmic membrane to become SITI (substrate internal transporter internal). SITI dissociates on the inner face to liberate SI into the cytoplasm and regenerate the transport element on the inner face as TI.

Various types of energy coupling, such as linkage to proton motive force and linkage to phosphorylation, are neglected. However, their effects may be approximated by choosing the rate constants $K1$ through $K8$ in such a way that the product of $K1$, $K2$, $K3$, and $K4$ does not equal the product of $K5$, $K6$, $K7$, and $K8$ as would be required if no other reactant and no other source of energy were involved. The substrate bound on the internal surface of the cytoplasmic membrane dissociates (or is made into a derivative) to become SI and is the primary transport product in the cytoplasm. SI is the organic species on which intermediary metabolism acts to generate the entire set of organic products needed for cell growth. In this scheme, a single reaction is used to simulate the multiple reaction that drains this substrate and leads to the formation of the multitude of required cell components. It is tacitly assumed that this reaction is the rate-limiting step and is the earliest reaction in the metabolic network. An alternative, equally suitable assumption, leading to the same kinetics, is that a control sensor occurs later in the metabolic pathway but affects an early reaction, possibly in the same way as feedback inhibition from a component at the end of a pathway segment controls the first step. Whatever the mechanism of control, this reaction limits how fast SI can be funneled off into cell constituents. The model approximates these effects by defining a maximum velocity, V', which I have modeled as a unidirectional Michaelis-Menten hyperbolically saturable process with a half-saturating concentration of KM. This is symbolized in computer terminology by the equation:

$$DCC = VPRIME*SI/(KM+SI) \quad (5)$$

The D in this and other examples in Table 1 signifies "taking the differential" in the sense of calculus, and CC stands for cell components in toto.

The first and second properties of the system that couples

TABLE 1. Heart of the computer program that simulates uptake and consumption[a]

```
DSE=−K1*SE*TE+K2*SETE
DTE=−K1*SE*TE+K2*SETE+K7*TI−K8*TE
DSETE=K1*SE*TE−K2*SETE−K3*SETE+K4*SITI
DSITI=K3*SETE−K4*SITI−K5*SITI+K6*SI*TI
DSI=K5*SITI−K6*SI*TI−VPRIME*SI/(KM+SI)−K11*SI
DCC=VPRIME*SI/(KM+SI)
DTIP=K9*SI*TI−K10*TIP
DTI=−K6*SI*TI+K5*SITI−K7*TI+K8*TE−K9*TI*SI+K10*TIP
SE=SE+DSE
SETE=SETE+DSETE
SITI=SITI+DSITI
SI=SI+DSI
TE=TE+DTE
TI=TI+DTI
TIP=TIP+DTIP
CC=CC+DCC
```

[a] This table is the implementation of the scheme shown in Fig. 1. D has the significance of d in calculus and means an infinitesimal difference.

uptake and growth is the key presumption that the internal transport product SI can "back-react" with the transport element. The dual actions of favoring either the reverse reaction or tying up the transport element would tend to slow net uptake to a value appropriate for the growth capability of the internal parts of the system. The third mechanism that might curb uptake and growth could be a mechanism that ties up the transport element by converting TI to TIP when the SI concentration becomes too high. TIP would be inert and does not circulate within the membrane so as to allow the entry of substrate. From our knowledge of prokaryotic control mechanisms, there would be many possibilities for such mechanisms, but I am really thinking that there could be something similar to bacterial two-component systems. In such systems, there is a sensor that causes the phosphorylation of an effector element in many instances. In the present case, the sensor is inside the cell and responds by favoring phosphorylation or possibly autophosphorylation of TI, leading to TIP, which is assumed to be incapable of transporting the substrate. This is symbolized in Table 1 by

$$\text{DTIP}=\text{K9*SI*TI}-\text{K10*TIP} \quad (6)$$

Lastly, the scheme includes an overflow pathway in which the internal metabolite, SI, in a first-order process, is metabolized to some other compound, which is then excreted. This is modeled in the scheme of Fig. 1 by diverting SI into two metabolites, one of which is secreted and the other is used for growth.

The final equation combining all factors affecting the change in the concentration of the internal substrate is complex and contains many terms:

$$\text{DSI}=\text{K5*SITI}-\text{K6*SI*TI}-\text{VPRIME*SI}/(\text{KM}+\text{SI})-\text{K11*SI} \quad (7)$$

The overflow process is idealized by the last term (K11*SI) in this equation. This equation includes both competitive product inhibition, since SI ties up the transporter, and noncompetitive inhibition, since the transport element is tied up in a nonfunctional derivative form, and it fosters overflow metabolism as well as funneling metabolites into growth processes.

Possible Uses of the Simulation

The portion of the program given in Table 1 just carries out a calculation of the changes in the amounts or concentrations of the many components of the model. To use it, one would have to provide initial values for all the components and the values for all the rate constants. For a given set of rate constants, the same values would be regenerated after one cycle if the proper steady-state initial values had been assigned. This is the case for all components except CC, which symbolizes the amount of collective cell components and will increase continuously. Such a computed constancy of the other parameters would be an indication that the parameters that yield a steady-state solution have already been found. Usually, however, the new value is different. For this reason, the program I use is arranged so that it allows a repetition of the calculation, causing a continuous readjustment of the various concentrations until eventually a steady state is reached. After the steady state is reached, such an approach, if continued in this way, would allow the growth of the bacterial system to be monitored. This would require taking into account the fact that the rate constants in this formulation are first-order pseudorates. Therefore, they are really second-order rate constants where the additional concentration is, or is proportional to, the cell biomass. This follows since the cell biomass must progressively increase during growth and, during balanced growth, be proportional to the concentration of a relevant enzyme. On the other hand, if we are interested in the steady state for some external concentration, this concentration, SE, would be fixed and the cycling would be continued until the internal concentrations of the various forms of T and of SI did not change.

The problem with numerical calculations of this kind is that the stable solution may require very many reiterations. If the problem is scaled up by multiplying all the constants by a factor or explicitly writing in the units of time in the equations and then choosing a larger value for the unit of time to be considered, in some cases the desired set of concentrations may be obtained quickly. However, in many cases, the solution may become unstable and generate infinite or negative values or even exhibit cyclic behavior. Although these difficulties can be avoided by using smaller units of time, the computation may require an unacceptably large amount of computer time for long-term phenomena like the cell cycle. Of course, if a personal computer is used, it could be allowed to work overnight or over the weekend.

The problem addressed in the previous paragraph is not a new one, and so there are many tools to solve such a system of "stiff" equations. A collection of mathematical tricks was assembled in 1971 by Gear (31), and they, or versions of them, are part of simulation applications available for all sizes of computers (19, 68, 69). Depending on the problem at hand, these tricks may be needed. However, here I will avoid using them so as to allow the readers to incorporate the program of Table 1 into a commercial simulation system or to most easily set up a computer simulation of their own without the use of any of the available elaborations.

The problem to be treated here is to find the way in which the transporter, T, is partitioned in the five forms of Fig. 1 under particular conditions. Redistribution of these forms of T takes place quickly because there is a low concentration of T in all its forms compared with the substrate flux and the biomass accretion. Consequently, the undulation in the values before they become constant are minor and can be handled in several alternative ways.

This paragraph is for the computer neophyte. For the purposes of such calculations, it is easy to put in a "while" loop. This means that the computer continues to cycle until the various forms of T and SI no longer change significantly, and then the cycling stops. The computer notes these steady-state values for the components and then tries another, successive, value of the parameter being varied, most usually SE. This cycling continues until the desired range of concentrations has been explored. Small time steps are usually adequate because the computers do their arithmetic to many decimal places. Therefore, even without a "while" loop, if the values of the different iterations are made to appear on the screen, it can be seen if the system is unstable. However, sometimes it is easy to average the peaks and valleys of the undulations and use these as initial values to quickly reach the steady state. Sometimes it is as easy to use the mathematical tools called regula falsi, simplex, or optimally multidimensional simplex (77, 91), which try various high and low input values and compute the limiting values very quickly. These methods find the desired answer in a systematic and efficient way.

Hopefully, the equations of Table 1 will be useful in studying uptake linked to consumption in conjunction with a variety of experimental studies. Thus, this section should be viewed as an advertisement.

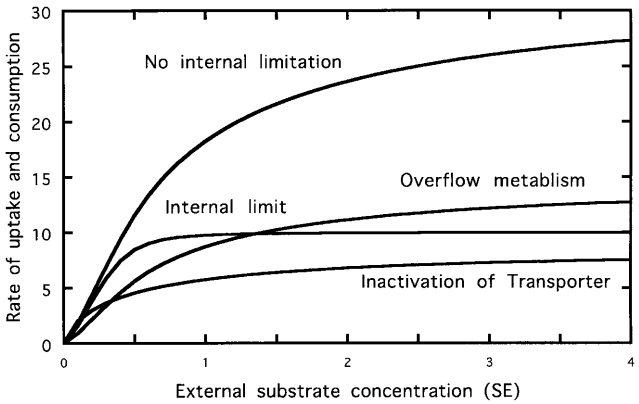

FIG. 2. Uptake coupled with growth. The computer program in Table 1 for the scheme shown in Fig. 1 was used for several cases. For the case of no internal limitation, the following parameters were used: $K1 = 0.001$, $K2 = 0.0001$, $K3 = 0.001$, $K4 = 0.0001$, $K5 = 0.001$, $K6 = 0.0001$, $K7 = 0.001$, $K8 = 0.001$, $K9 = 0$, $K10 = 0$, $K11 = 0$, $V' = 0.001$, KM = 0.001, CC = 1. The program evaluated the rate for a total content, T, of 0.1 and various values for the external substrate, SE. For the case of internal limitation V' was set equal to 1/100 of the standard value. This made the synthesis of cell intermediate metabolites and macromolecules limiting. This is almost the case envisaged by Best (3), discussed more extensively in the text and in references 51–53, 56, and 58. For the case of inactivation of transporter, the program parameter $K9$ was set equal to 1,000 and $K10$ was set equal to 0.001. For the case of overflow metabolism, $K11$ was set equal to 0.1 and V' was set equal to 0.0001; otherwise, the original set of parameters was used.

APPLICATION TO VARIOUS CONTROL SYSTEMS

In this section, the computer simulations will be used to show the effectiveness of the various types of regulation of the uptake processes by growth limitation. With an arbitrary choice of values for all the parameters, a program based on Table 1 generated a curve of substrate uptake rate (equal in the steady state to the rate of utilization for growth) versus substrate concentration.

First, the simulation for a single substrate is shown in the top curve of Fig. 2 for the case marked "No internal limitation." For the parameters chosen, the maximum utilization rate for growth was high enough to not impede intake and to retain the transport kinetics as the limiting stage of growth for all SI values. For this simulation, the parameters and initial values are given in the legend to Fig. 2. It can be seen that the uptake is nearly hyperbolic. There is a slight foot on the curve, and the Hill coefficient would be slightly greater than 1. Considering how many parameters are involved, one might have expected a much more complicated substrate dependence curve. However, the relative simplicity of the curve results from the cyclic way in which the transporter system functions.

Control by product inhibition is an inherent feature in the remaining curves, and the curve labeled "Internal limit" has no additional control other than a limiting V'. If the internal substrate concentration can rise to the point that the transporter on the inner face is forced to recombine with SI, influx will be slowed because of the decrease of TE. The only change made in the parameters is to decrease V' 100-fold. By comparing these two curves, it is clear that the apparent K_m is markedly less for the internal-limit curve. This is true even though no changes were made in the total amount of transporter or the reaction rate constants for the membrane-linked reactions. This contrast in apparent K_m points out a problem that has caused much confusion in the literature, i.e., when workers did not realize that the apparent K_m need not reflect the kinetics of the actual transport process. This is reflected in the variety of K_m values in the literature reported for the uptake system of *Escherichia coli* for glucose.

Regulation might also occur reversibly as shown by the curve marked "Inactivation of Transporter" in Fig. 2. This additional or alternative process for the linkage of uptake to the ability of the cell to grow is some special control functioning by reversibly tying up the transporter element. This could possibly be akin to a two-component system. Here, such a mechanism is idealized so that SI controls the rate of conversion of TI to TIP by a first-order process. An alternative possibility is an allosteric type of control, but this is only one of several other possibilities. However, the point is clear: mechanisms removing transporter from circulation can effectively link uptake to regulate growth and decrease transport well below the level at which the transport system could maximally function. Such mechanisms would decrease the apparent K_m.

The final way in which the ability of the cell to grow is linked to uptake is shown by the curve marked "Overflow metabolism." In this case, the internal substrate is metabolized but some reaction product is excreted into the environment in a changed form. I have not used a metabolic scheme that shows the classical splitting and extrusion of one component and the utilization of the other component for growth, but this is not important for the argument here. For simplicity, I chose the case where SI could be changed to another metabolite and excreted in a first-order process. The curve marked "Overflow metabolism" shows that this can be an effective means of limiting growth when a regulatory system of this kind is in place and it is important to the cell to do so. This process does not greatly affect the Michaelis-Menten constant for uptake by growing cells.

Finally, it should be noted that the interpretation given here for overflow and energy-spilling metabolism is only somewhat different from that subscribed to by Neijssel and Tempest (76, 77, 104) that the process reflected the balance of diverse metabolic capabilities: transport is stressed here, whereas they stress certain parts of intermediate metabolism. The point of all four of these regulatory processes is that they function in a way that lowers the concentration of the substrate inside the cell when consumption cannot lower it and thus may tend to protect the cell from osmotic stress or from actual poisoning by the metabolites.

SIMULATION FOR MULTIPLE SUBSTRATES

If the reader accepts the formulation and modeling of the uptake and coupling to cell growth made above, an important extension to two similar but different substrates can be applied. This is not the place to consider the phenomenon of diauxie that Monod studied for his Ph.D. (71), but it is the place to consider the coutilization of substrates in the absence of changes in enzyme induction (28, 29, 64, 75, 93). For this extension of the simulation in Table 1, all that we need to do is duplicate the kinetic system of Table 1 with different suffixes (say, A and B) for the symbols representing constants and components of the two substrates. The linkage between the two would be simply that the V' for one substrate plus the V' for the second must add up to a fixed quantity, i.e., the global V', because the idea is that some internal process of macromolecular biochemistry within the cell finally governs the maximum rate of cell growth.

With so many choices of values for the parameters, a variety of growth patterns can be simulated. For example, one carbon compound might prevent the utilization of another, or they both might be used in proportion to their concentrations outside the cell. Only one example is shown in Fig. 3. This is for

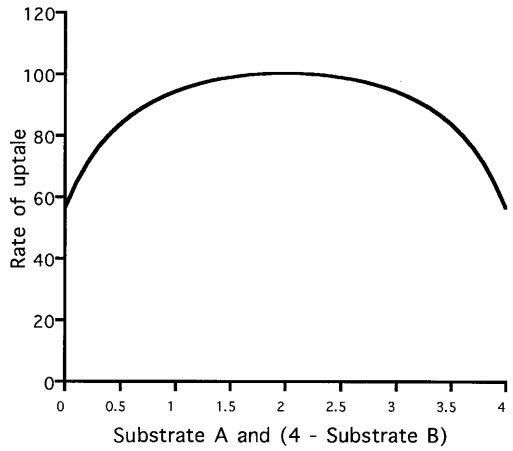

FIG. 3. Coutilization of two substrates. The program was augmented by considering two totally independent uptake systems for two different kinds of substrate. Once inside the cell, the program permitted either substrate to be equally used for growth. Because the ability to grow was limited by the total V' for growth, which depended on flux derived from both sources, a combination of the two resources supported a higher growth rate than did either alone.

the case when neither substrate is present in large enough amounts to saturate the metabolic needs, and so when both substrates are present the total uptake and growth rate are greater.

Experimental work from Egli's laboratory (28, 29, 64) is currently extending the earlier mixed-substrate studies by Silver and Mateles with chemostat cultures (101) and those by Hegewald and Knorre with batch cultures (35). Other kinds of theoretical developments are being contributed from the laboratory of Ramkrishna (75, 105). The approach of Ramkrishna's group is a "cybernetic" one, which assumes that the cell has an unspecified way to alter the rates of different enzyme systems to optimize its growth under any particular conditions.

Possibly more significant are developments that will allow the experimental study of multisubstrate cultures without the use of chemostat cultures but instead by studying very dilute cultures. Such experimental paradigms, more relevant than chemostat culture, are being developed. By using a tuned-up and well-aligned flow cytometer, it is possible to measure the number of cells when they are below 10^5 per ml. Recently, it has become possible to convert the channel number of the flow cytometer into a measure of cell size (59). This means that it is possible to measure the mass growth rate of cells at very low concentrations in arbitrary mixtures of substrates under conditions where the concentrations are well known and essentially constant because the concentrations of bacteria are so low that they never perturb the substrate concentration. Consequently, it would be possible to measure experimentally a curve like that shown in Fig. 3. Alternatively, it would also be possible to perform such experiments with an instrument based on the Coulter principle. In fact, Shehata and Marr (99) did exactly that 25 years ago.

THEORY OF DIFFUSION TO THE CELL SURFACE

To understand diffusion to the cell surface, we start with ancient history in a quite different field. In the 1930s, Schlesinger (98) studied the rate at which viruses infected bacteria and found that it was so high that it appeared, for his case, that every collision of a virus with the target cell led to an infection. For the theory to match his experimental results, he used Fick's diffusion law. Later, Delbrück (22) found that sometimes not every collision led to infection, and he proposed, still based on Fick diffusion law, that the rate of infection was

$$dq/dt = 4\pi DCa \quad (8)$$

where dq/dt is the rate of absorption on the surface of a spherical cell target, D is the diffusion constant of the virus particle in the environment, and C is the concentration of particles at a long distance from the cell, i.e., in the bulk of the medium. If S is the concentration of particles at a distance, r, from the center of the spherical cell, he was able to show that

$$C = S(1 - a/R) \quad (9)$$

The distance a, nominally, is the radius at which C becomes zero and is equal to the cell radius, R, if every collision resulted in infection and is equal to some smaller distance if not every collision resulted in infection. Because experimentally R appeared to be equal to a, it appeared to Schlesinger that every collision was effective, or so he claimed (98). Koch showed in 1960 (45) that if a was just a very small amount less than R, very many collisions could occur and effectively it would appear that the spherical cell was absorbing every virus that collided with it. In 1977, Berg and Purcell (1) showed the same thing in a much more elegant way. They demonstrated (see also references 2 and 92) that if a in the above equation was replaced by $RNs/(\pi R + Ns)$, where s is the radius of each absorptive site and N is the number of the absorptive sites on the surface of a spherical cell, only a very small number of sites sufficed to make it appear that every collision was fruitful, i.e., as if the whole surface was perfectly absorptive.

Translating the above discussion to the present context means that if only a very small percentage of the cell surface consists of regions where uptake of a nutrient is possible, every time a substrate molecule collides with a cell it is very likely to be eventually taken up and not to diffuse away. Even if the collective area of porins or permeases is very small, this occurs because the substrate will remain in the vicinity of the cell long enough that it will most probably have a fruitful collision before it diffuses a great distance away by chance. This logic led me on a quest to find how efficiently *E. coli* could adsorb a very good substrate, i.e., glucose, when present in a very low concentration where diffusion up to the cell was necessarily limiting.

UPTAKE AT LOW SUBSTRATE CONCENTRATIONS

There are a number of steps (see Fig. 4) for a molecule of substrate, initially at a long distance from the cell, to undergo before being taken up through the cytoplasmic membrane. It is known that travel through the cytoplasmic membrane is the major rate-limiting step at low substrate concentrations, but passage through the porins is a partial and important limitation.

The quest to study growth at low substrate concentrations led to the design and use a supersensitive and accurate spectrophotometer linked to a computer (51, 52, 60, 110). It was found that dilute cultures of *E. coli* growing at very low glucose concentrations depleted the culture of glucose and the growth rate fell gradually. The observed kinetics satisfied the Best equation better than they satisfied the Monod or Blackman equations.

If one rearranges equation 8 to calculate the maximum efficiency of uptake of a spherical particle, i.e. when $a = R$, it is found that the maximum efficiency, E (the number of cell volumes taken up by the cell per second), is given by $3D/R^2$. For a cell with the volume of *E. coli*, the expression $3D/R^2$

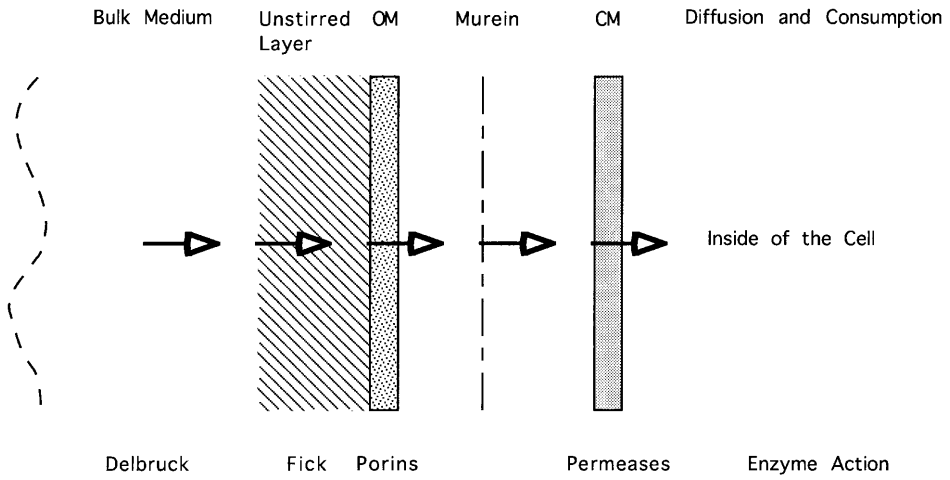

FIG. 4. Stages of diffusion in the flow of a substrate through various regions and through various impediments in the environments and within the layers of the cell membrane of a gram-negative bacterium. All these stages are considered to be very rapid for the scheme and modeling of Fig. 1 to 3.

shows that the theoretical maximum efficiency is 3,280/s. Experimentally, the efficiency was found to be 72/s in this first-order, low-substrate concentration region with batch-grown cells in ordinary glucose-M9 medium. However, when the cells had been grown in a glucose-limited chemostat for a month, the efficiency rose about ninefold to 651/s. Of course, *E. coli* is a gram-negative organism with an outer membrane acting as an impediment to uptake and growth. Passage through the porins is truly an impediment, even though glucose is small enough to pass through the pores of the porins; in fact, diffusion through these pores contributes a kinetic limitation to growth.

The maximum efficiency calculated above would be the expected value if the cells had had their outer membrane dissected off and if the cytoplasmic membrane was completely covered with phosphotransferase system sites. The probable reason why the glucose-limited chemostat-cultured cells were more efficient than the batch-grown cells is that the C-type porins were replaced by the larger F-type porins. This would account for the observed increased efficiency, and an analysis of the mathematics of diffusion through pores of different radii (i.e., the C and F types) gave results consistent with the observed ninefold increase (60). However, an outer membrane containing only F porins would still slow diffusion enough to account for the fivefold (3,280/651) factor. Thus, if there were many porins and they had very large pores, the efficiency would be further increased approximately by this factor. It can be inferred that the effective transport through the cytoplasmic membrane of the cell is as efficient as the laws of physics of diffusion permit.

Compared to the case of chemostat-grown cells with no outer membrane or one with a very-high-porosity outer membrane, the cell had put itself into a 45-fold less efficient condition (3,280/72) as it grew in excess glucose than if the naked cytoplasmic membrane were exposed to the medium. It seems that *E. coli*, or any gram-negative cell, maintains and retains an outer membrane because the membrane plays a very important role in the total biology of the organism. Evidently, the outer membrane is not something that the gram-negative organism is able to live without; probably, the many hydrolytic enzymes that supply the cell with consumable nutrients are important factors. Note that during our laboratory culturing, we had not subjected the organism in either batch-growth or chemostat-grown cultures to any known positive selective pressures to retain the outer membrane. None of the purposes which are usually suggested as the function of the outer membrane are relevant in these experiments. Those that are usually suggested for the existence of the outer membrane include preventing the entry of antibiotics, bile salts, or bile acids and the need to retain hydrolytic enzymes near the cytoplasmic membrane to provide smaller molecules for nutritional purposes.

PROBLEMS WITH CONTINUOUS-CULTURE STUDIES

There are two types of continuous cultures in use; these serve different experimental purposes. I am not drawing a distinction between a single growth limitation and non-nutrient-limited cultures. In the first class here, I include both the chemostat of Novick and Szilard (80–82), the Bactogen method of Monod (73), and the turbidostat of Bryson and Sybalski (6). They were designed to help elucidate bacterial physiology and bacterial genetics. In the second class, I include the modern commercially available laboratory fermentation apparatuses. They are prototypes for a possible manyfold scale-up to be used in large-scale fermentors in the pharmaceutical industry. The biomass concentrations in the two classes are quite different. Ordinarily, the biomass concentrations for physiological purposes are well under 10^9 bacterial cells per ml, but for commercial purposes they are well above this value. For the former type, heat production is small and essentially irrelevant, but adequate mixing may be a problem. For the latter, oxygenation, pH, heat removal, and temperature control are crucial. Because of the higher biomass concentrations in the second type of culture, the interaction between cells is more important, whereas in the first type it is important to use conditions where the cell-cell interaction is insignificant, because the goal is to study the physiological and genetic processes within a single cell isolated from the actions of its neighbors.

Continuity of Flow and Mixing

When the chemostat is used to study the physiology of slow growth and therefore lower flow rates are used, the effect of dropwise addition of culture medium and the effectiveness of mixing must be determined. During chemostat growth, the

conditions are adjusted so that some nutrient is limiting. This is not to say that the cells are starving. Ideally, there is always some nutrient, just never enough. To make a clear interpretation, the constancy of the substrate concentration both from place to place within the culture vessel and from time to time is essential. This is not a factor for the industrial studies with laboratory fermentors, where mixing is so violent and rapid and the flow of medium and air is so great that the constancy of substrate concentration is likely to be good. Because the uptake systems can be fast, perhaps a few seconds of substrate deficiency or excess due to the mechanics of the growth machine might be very important to the organisms, even if the average external concentration were correctly estimated by the well-known equations for chemostat growth given in most elementary microbiological tests, i.e.,

$$dN/dt = \mu N - (f/v)N \quad (10)$$

where N is the number of bacteria per milliliter and dN/dt is the growth rate, assumed to be identical throughout the vessel. The growth rate constant, μ, is usually assumed to depend hyperbolically on the free substrate in the culture vessel although the Best equation is usually more accurate. The other symbols in the equation are f, the flow rate, and v, the volume of the chemostat culture. Because of the possibility of significant fluctuations of substrate concentration, I had been greatly troubled that the chemostats that I and Tom Norris designed (47, 79) might be inadequate. If this were so, the results that we had published with the apparatus might be incorrect because they might not meet the criteria inherent in the assumptions which lead to equation 10. This equation is based on the presumption that the growth medium flowing into the culture vessel is immediately and completely mixed and that the bacteria consume the substrate so that the free concentration is rigorously constant throughout the chamber. We (49, 50) had to do experiments and calculations to convince ourselves that, from the point of view of the bacteria, the conditions are effectively constant.

The questions we asked were how large a supply of glucose could a cell sequester when the cell was right under a falling drop in the chemostat and whether the glucose would last to be used later when no external supply was available. To test these questions, two chemostats were operated simultaneously with strains that differed in only one gene, the gene that allowed β-galactosidase to be induced (49). Aliquots taken directly from the chemostats were mixed with a small amount of glucose and with the gratuitous inducer isopropyl-β-thiogalactopyranoside (IPTG) in a timed series so that either the inducible strain or the noninducible strain had a longer time to pump in the glucose in the absence of the other strain and could therefore deplete the medium before the other strain was introduced. In this way, one could estimate how long-lasting a supply of glucose could be taken up to be used subsequently for β-galactosidase formation. For our conditions (47, 49), the cells could take up 18 s worth of glucose beyond their current needs. To restate the conclusion, when glucose was available, uptake led to the sequestering or hoarding of a resource inside the cell in a form not yet used for cell growth. It would appear that the metabolism of the cell would be continuous as long as the cell in culture was circulated into a region with low levels of glucose every 5 to 10 s.

This could be compared with the much shorter mechanical mixing time of our apparatus. This was measured with the same chemostat but without bacteria or culture media. To estimate the mixing time, the chemostat apparatus was set up as follows: instead of bacterial culture, the vessel contained some dilute acid and phenolphthalein indicator, and the solution being dripped in was alkaline and also contained phenolphthalein. By a sparging action, the pink drops would be rapidly mixed in the colorless fluid of the chemostat vessel, but only when the mixing was fully complete would the color fade. The mixing time in our situation was 3 or 4 s, much shorter than the hoarding time of 18 s. On this basis, we could justify our conclusions about the physiology of the cell.

It should be pointed out that a series of similar tests must be carried out with every different apparatus and a range of experimental conditions.

Measurement of Substrate within the Culture Vessel

Measurement of the substrate concentration within the chemostat is difficult. There have been improvements, including stopcocks that can be opened quickly to allow a sample to be expelled into a quench solution to stop metabolism and also ways to quickly process a sample for analysis by membrane filtration. However, defining how many milliseconds of uptake still occurred as the sample was taken and mixed with a quenching solution or during separation on the filter is difficult. Moreover, distinguishing between substrate in the medium and substrate within the cells which might be returned to the medium in the quenching process is not trivial. Additionally, knowing whether a particular procedure is quick enough to not invalidate the conclusions is even more difficult. This may be especially important, for example, in studies of multiple substrates. A chemostat that was combined with a stop-flow machine would probably be adequate to measure the free concentration in most cases.

Wall Growth

The bane of chemostat work, particularly for ecological, genetic, and evolutionary studies, has been wall growth, i.e., growth of the cells attached to the walls and surfaces of the culture vessel. There is no doubt that many published studies with chemostats and turbidostats can be faulted because of wall growth. Various methods have been tried to eliminate it; these include windshield wipers to clean the glass, the use of Teflon in construction of the chemostat vessel (47), the use of a very large culture vessel so that the effect of cells attached to the glass is reduced (47), and, finally, transfer of the contents of the culture vessel into a new, clean culture vessel. Certainly, the most successful device is that of Marliére, in which the culture is transferred to a new vessel at intervals, the old vessel is sterilized with strong base, and later in the cycle the culture is returned to the first vessel and the second is resterilized (65). This technique has allowed the study of evolution in an uncontaminated culture for periods of years without complications due to wall growth.

Foam

As with wall growth, a corresponding difficulty arises because many bacterial cells partition at the air/water interfaces with a higher concentration of cells in the foam that results from strong aeration than in the liquid. While antifoaming agents can be used, their effect on the organisms is not known. Another way to avoid the problem is to have the outflow not remove foam at all but only the liquid part of the culture (47).

Future Use of Continuous Cultures for Physiologic, Genetic, and Evolutionary Studies

The above comments have been critical of the use of continuous culture in physiologic studies because of flaws in the

design of the equipment and/or in the execution of the experiments. Of course, very useful results have been obtained with such devices, but it is necessary to keep in mind the difficulties described above. For the critical study of cell processes during slow growth in continuous culture in the future, these difficulties must be fully overcome. I know of only two ways that overcome them and achieve an essential perfect steady state, but I do not believe that either has been exploited.

One was an idea formulated and exploited in a limited way by Jacob. He knew that *E. coli* had no invertase and could not metabolize sucrose. He therefore studied a series of cultures with sucrose as the sole carbon source and supplied various levels of yeast invertase to generate glucose and fructose distributed throughout the culture vessel. Because the consumable sugars were created at a very large number of sites in the vessel and consumed at a very large number of sites within the culture, the steady-state assumption is certainly likely to hold critically. His old design could be brought up to date and combined with a continuous-culture device. Such an apparatus would have not only an input flow of growth medium containing sucrose but also another input with a very low-volume flow of a suitable concentration of a sterile solution of invertase. This procedure would be as if a chemostat were used with millions of orifices that introduced new medium throughout the culture volume.

The second possibility is to feed the nutrient into the vessel not as drops or a stream but as an input to an Amicon-type device that has many thin and porous tubes radiating from the input source. The other end of the device would be stopped up. Culture medium would continuously seep out of the length of each tube and thus greatly decrease any fluctuations in the concentration of substrate at any point in the culture. These are the only ways that I have been able to find to allow a rigorous study of possible cellular control mechanisms for uptake of substrate and growth of cells in a well-defined uniform state of balanced steady-state growth.

MACROMOLECULAR SYNTHESIS MAY BE CATASTROPHIC UNDER SEVERE CONDITIONS

Cellular Response to a Surfeit of a Resource

When a starved cell is refed, there often are problems. The cell may not survive or at least may not return to active growth immediately. Consider a nonextreme example when glucose-limited chemostat cells were provided with an excess of glucose. They grew faster immediately but took 6 h to reach the expected steady-state growth rate for the new concentration (49). On the other hand, when shifted up to a very rich medium containing both the components of Luria broth and M9 minimal medium (this mixture is the precursor of Terrific broth), the cells took just a few seconds to reach a definitive steady state with a very high growth rate (a doubling time of less than 20 min). This startling result suggests that the cell in a balanced state of chemostat growth had activated some mechanism to protect itself against a sudden influx of glucose and has only slowly relaxed it. Such a blockage may have been essential during growth in the chemostat with a pulsatile, second-by-second exposure to substrate. On the other hand, the mechanism prohibiting rapid fluctuations in the level of certain intermediates did not act on many other compounds, so that the transfer to the Luria broth/M9 medium could cause an immediate shift up to a very high growth rate.

Oligotrophy, VBNC, and SAD

This result of bacterial "reticence" described in the last section is reminiscent of the behavior of oligotrophs (sensu Poindexter [87, 88]). Oligotrophs can grow under conditions of low levels of nutrients but may not grow at high levels. Other phenomena that may be related to this are substrate-activated death (15, 89), killing by lactose of a constitutive variant selected in the chemostat that had been limited by lactose (25, 50), and the currently popular viable but not culturable state (18, 83, 102). The commonality in all these processes may be due entirely or partially to an inability of bacteria to deal with a surfeit of carbon compounds internally. This could be due to the pool of carbohydrate swelling manyfold and leading to cell death from osmotic stress caused by swelling and leaking or rupturing (57).

A Need To Be Able To Complete a Macromolecule Once Begun

Almost 30 years ago, we observed (58) (also see the section on quiescent but not sporulated or encysted cells, below) that slowly growing cells in a chemostat are heterogeneous in their behavior although they are still in a steady state. Although all the cells have the ability to synthesize protein, some of the cells were not engaged in protein synthesis at any given time. The implication is that the cells have a choice about their global synthesis of protein and energy expenditure and that in a carbon-stressed environment they sometimes do not attempt to synthesize a protein if they do not have enough resources to finish making the macromolecule. Having all the ribosomes in the cell engaged in protein synthesis could be dangerous when there are inadequate resources to finish the started molecules. Partially completed proteins may be subject to degradation, as would the stalled mRNA or partially opened and effectively denatured DNA. It is known that incomplete and misformed proteins are more rapidly degraded. It is also clear that starting but not finishing a round of DNA replication would be counterproductive to the health of the cell. It is known that thymine starvation leads to mistakes resulting from stalled DNA synthesis that may cause death and/or higher mutation rates. This suggests that death or higher mutation rates when DNA replication is temporarily stopped may be caused by carbon starvation.

OTHER UNRESOLVED ISSUES FOR SLOW GROWTH OF BACTERIA

Maintenance Energy

To account for the yield of bacteria in chemostat cultures as a function of the dilution rate, Pirt (84) and Marr et al. (66) simultaneously developed the concept of maintenance energy. The idea is in some sense trivial: analogously to an internal combustion machine, fuel and air are needed to power the work that the cell is to do, but it needs additional energy and resources to repair and replace worn parts; and it may have to waste energy (idling) to keep the wheels turning. However, there are additional reasons for maintenance energy: the cell may need ways to dissipate unneeded energy that may be thrust upon it by the environmental conditions such that the energy cannot be immediately put to use, and it may need processes that require continuous energy consumption, just so that the cell is in a position to be able, if needed, to alter its metabolic course. (This logic was one of the original ideas that led to the discovery of mRNA. It had been found that *E. coli*

could start and stop β-galactoside production very rapidly. This suggested that short-lived informational molecules with a rapid turnover had to exist. This is, no doubt, a very important justification for the expenditure of maintenance energy.)

Another reason for maintenance energy is that the cell may need ways to maintain surveillance of the environment to guard against other bacteria, viruses, toxic chemicals, etc., and therefore has a multitude of processes that may appear to the physiologist or biochemist as futile cycles. The term "futile cycles" describes the concept arising from situations in which the cell has both an irreversible pathway for a chemical transformation process in one direction and another different and irreversible mechanism for the reverse process (it can also happen that two processes may simply have greatly different K_m values which together can serve to dissipate energy). If one or the other functions at a different time, metabolism could be efficient and not wasteful. However, if they did act simultaneously, there might be no net transformation and the only result would be that cellular energy is dissipated. The physiologist's understanding of "futile" is slightly different, in that it has the function of allowing the cell the opportunity for rapid change.

However, the list of possible reasons for maintenance energy expenditure has only begun. Another reason is the following. Because there are environmental fluctuations, many bacteria have evolved ways to protect themselves by shutting down metabolism and waiting, as in, for example, the well-known endospore formation by bacilli. However, many alternatives may serve the same purpose in other members of the microbial community that do not sporulate. All these alternatives depend on potentially wasting some resource while protecting the longevity of the species. No matter what the cost of forming spores or the other forms of mothballing, there may be a significant cost if the organism misses opportunities to use resources and to grow. An example could be if the cell is in a resting state when conditions become favorable for growth. This is epitomized by the first postulate of microbial ecology, which is equivalent to "If you are asleep, you won't get dinner."

Much of this would seem to be anthropomorphizing or philosophizing by Pirt (84–86), Marr et al. (66), and Koch (49, 53) and still needs to be justified when applied to microbes generally. However, the list of reasons for maintenance energy is still not complete. For the chemostat cultures at different dilution rates, there may be fictitious contributions to maintenance energy. We therefore need to add the following additional sources of apparent maintenance energy: wall growth, which adds cells to the physical surfaces of the vessels and subtracts them from the culture fluid, and enrichment of bacteria in the froth in the culture vessel, which removes an excess of cells, leaving the culture fluid deficient in cells. These circumstances have been briefly discussed above.

Overflow Metabolism

Bacteria are like Americans (and other humans): we waste resources, rather than conserve for the future, if it is more convenient for us as individuals, groups, corporations, or governments to do so. Overflow metabolism, the intentional wasting of resource, has also been designated by terms like metabolite spilling, spillover, and metabolite overproduction (77). The positive value of such a metabolic process is simple if we anthropomorphize and argue teleologically. The argument is that the organisms are acting on the expectation that the local resource is part of an indefinitely large and continuing supply, and the philosophy is "eat, drink, and be merry, for tomorrow we die." Consequently, if only partial utilization of the resource molecule is more effective, even though more is required, the profligate behavior will be positively selected. On this basis, the cells may transform and split the molecule to a limited degree and excrete it. Thus, a portion is thrown away, as an alternative to complete consumption, because it is more cost-effective. This is a metabolic choice even though these cells are capable of complete utilization. This conspicuous consumption and the throwing away of a useful residue is an economically defensible mode of action from the perspective that it is faster, easier, and more efficient. In many such cases, the residue is a resource for other organisms or mutant strains (53, 95).

Consider two examples, the incomplete consumption of glucose with the extrusion of acetate (95), and the incomplete consumption of glucose with the extrusion of α-ketoglutaric acid and/or glutamic acid (53). Both processes are in some sense evolutionarily selected and are modified or optimized in different natural strains that survive in different ecosystems. The advantage of prudent complete consumption over partial use by omniscient bacteria would depend on a much more global knowledge than an actual individual organism could possibly have. However, an ability for overflow metabolism is built into many organisms and can be activated or quenched by mutation. Physiological control of such mechanisms has scarcely been studied.

Energy Domains

Under conditions where energy availability is the critical limitation for the organism, the organism is placed on the horns of a dilemma of how to optimize its energy use strategy. Chesbro and colleagues (16, 17, 107) conducted experiments in a flow system in which a culture is recirculated and passed over a membrane filter that removed the cell-free fluid. The fluid volume is replaced by new nonnutrient solution. In this system, the specific growth rate of the organism becomes progressively lower and lower, and it was argued by Chesbro and colleagues that the cell population changes en masse in discrete steps into cells functioning with different energy strategies. This is reflected by different levels of "magic spots," i.e., ppGpp and ppGppp, and the different cellular yields from the resource. This research would argue that there are multiple states of slow growth.

Threshold for Active Metabolism

The response of cells to starvation seems to depend on the time course of depletion of energy resources. If the cells are abruptly deprived of a nutrient, they may have enough reserves to enable them to synthesize mechanisms to permit survival. However, it can easily be shown that new protein synthesis is needed to shut down (23), to mothball (47, 49), or to sporulate. Otherwise, having once turned off metabolism, there is a problem of awakening. Relevant experiments include those of Button (9). His experiment was to take seawater that appeared perfectly clear and amend it with a very small amount of a radioactive amino acid. His group found that there was a threshold for its uptake by the microorganisms present in the water. When there was too little of a utilizable carbon substrate, the few cells present did not take up any radioactivity. If there was an amount larger than a certain critical level, uptake occurred and the rate of macromolecular syntheses was proportional to the higher level. This suggested that these organisms are truly metabolically inactive, unless an adequate level of an appropriate resource is supplied.

Quiescent but Not Sporulated or Encysted Cells

Koch and Coffman (58) found that when *E. coli* cells were cultured at low dilution rates in chemostats, some of the cells were not synthesizing protein while other cells were doing so at any given time. To demonstrate this phenomenon, Koch and Coffman measured β-galactosidase during pulse induction experiments. One assay was the usual one in which the bacteria are lysed with sodium dodecyl sulfate, but the second assay was quite different—it involved the hydrolysis of *o*-nitro-β-D-galactopyranoside (ONPG) by dense suspensions of permease-negative intact bacterial cells measured in the double-beam spectrophotometer. This assay depended on the distribution of the enzyme in the cellular population. In cells with a high enzyme concentration, the hydrolysis would be limited by slow permeation of the substrate to the inside of the cell through the cytoplasmic membrane into the cytoplasm where the enzyme was located. If the induced enzyme was uniformly distributed in all cells, all the enzyme molecules would have equal access to the substrate from the medium and hydrolysis of ONPG would be more rapid than if the enzyme was distributed in only a few of the cells. Consequently, when the enzyme was concentrated in a few cells, diffusion through the area of the cell membrane of these cells was limiting and there was less apparent enzyme function.

With this test, it was shown that all cells in chemostat populations of the inducible *E. coli* ML 30 growing with doubling times up to 13 h had a uniform and rapid response to inducer. On the other hand, cultures growing more slowly were heterogeneous. Some cells were temporarily quiescent (possibly accumulating reserves), but all the cells self-activated and synthesized proteins at the normal rate at some time during a 3-h period. It was found that the step time for amino acid addition to a growing peptide chain was substantially the same as that characteristic of cells from a more rapidly growing chemostat culture or from cells growing in a nonlimiting medium which otherwise had the same composition. Thus, the cells that were in the synthesizing mode were adding amino acids at the normal rate. For a 24-h doubling time chemostat culture, only about one-third of the cells were actively engaged in protein synthesis any instant in time. This type of heterogeneity could not be due to inadequacy of mixing in the chemostat culture. It is probably controlled by minor fluctuations in the energy reserves or intermediate metabolites (amino acids levels affecting the stringent response) available to individual organisms and depends on the time since the last activation of protein synthesis. Similar to our studies, Kell's laboratory (39–41, 109) found dormancy relationships in *Micrococcus luteus* cultures. Therefore, the quiescent cell represents a very temporary equivalent of the shut-down state described by Dow et al. (23).

PUZZLES FROM THE ECOLOGY OF SLOW GROWTH

Need for Thermodynamic Brakes

The principle of microscopic reversibility is a key principle in chemistry. It asserts that if a reaction can go forward, it can go backward. From this concept, it follows that a catalyst, such as an enzyme, can only speed a reaction to equilibrium. Intermediary metabolism functions by cells having only certain enzymes and not other conceivable enzymes. If cells do not have certain enzymes and with a net flux of free energy through the system, it is possible for less stable compounds to be made from more stable compounds. The converse of this well-known argument is that when no metabolic energy is available, equilibrium should be eventually established and the composition of the pool of intermediates should approach that of the thermodynamically most stable assemblage. This, of course, is a nonliving state containing no informational macromolecules.

The implication is that during starvation, hydrolytic enzymes should destroy many cell macromolecules and produce amino acids, nucleic acid bases, monosaccharides, etc. While there are a few cases where cells autolyze (*Bacillus subtilis* and *Myxococcus xanthus* come to mind), most do not or, at least, do not do so very easily. Since lysis does not happen when most cells are deprived of energy, the question is, "Why not"? An analogy to an automobile comes to mind. Imagine driving a car up a mountain road and running out of fuel. The simplest imaginable car would roll back down the hill, because it has no gears or brakes, but a modern car would not. Moreover, an actual car has compression due to the coupling of the wheels to the cylinders, which would slow movement. Also, the driver could put blocks under the wheels, or with the dying gasp of the engine, the car could be straddled across the road. The bacterial versions of setting the brakes and preventing "downhill movement" is more subtle. For a cell like *E. coli*, when resources gradually become scarce, cell division dominates biomass production until a smaller cell with only a single genome and few ribosomes results. Although such a cell can resist starvation for weeks (47, 49, 100), the issue here is that the synthetic machinery does not appear to act reversibly. This is partly because the processes are so complicated that at each of many stages of metabolism, the highly specific action of enzymes could slow the reversal owing to the absence of energy sources. It probably would be worthwhile to analyze the mechanism of these processive processes, and we could hope to find that the ultimate equilibrium could be achieved only extremely slowly by a direct reversal of the synthetic route when energy and metabolite conditions become limiting.

Strategies for Survival when Growth Is Not Possible

Starvation biology is now a very active field, with the Matin (67), Kolter (61, 100), and Hennge-Aronis (36) laboratories developing the molecular biology. Suffice it to say that there are complex mechanisms, still largely unknown, at several levels that function to keep the cell in a mothballed state of suspended animation. However, a quite different and relevant approach involves studies of mutation rates and processes in starving cultures. This approach was started with the provocative paper by Cairns et al. (14) that suggested that mutation in the starving state could be "directed." Through the insightful experiments of Hall (32) and the follow-up and extension of the Cairns approach by Foster (30), it is becoming clear that a starved cell has many mechanisms that serve to promote survival even when the cell is not capable of converting itself to some type of spore or resistant form.

CONCLUSIONS

The metabolic regulatory connection between the ability to grow and uptake capacity has been explored in this review. It is concluded that some cells under some conditions have the capacity to import too much substrate from the environment and suffer as a result. In many instances, the surfeit of substrate is deleterious to the organism. However, many bacterial cells have ways to limit the internal concentration of molecules, and much microbial physiology remains to be learned about this ability and its implications for prokaryotic cell biology. Microbial physiologists need to understand these phenomena, which are due to too rich an environment and exploit them. At the same time, the problems resulting from growth limited by the

deficiency of nutrients from the environment and during enforced growth at low rates require much serious study.

Different processes during the growth of bacteria take different periods to reach a steady state. The time for the transport machinery in the cytoplasmic membrane to reach a steady state is a fraction of a second. The time to fill the metabolic pool is a few seconds. The time to achieve balanced and/or exponential growth is a few hours. The time to adapt to the limitations of an ecosystem is minutes to days. The time to evolve the myriad of genetic tricks (mostly not expressed while growth conditions are adequate) is less than 3 billion years. Some of these protective mechanisms may function to protect cells from temporal fluctuations in the availability of resources that have a response time from seconds to years. Conversely, many cases can be documented where the fluctuations exceed the protective mechanisms of the cells and in these cases are dangerous or lethal to microorganisms.

ACKNOWLEDGMENTS

My interest in the importance of transport into the bacterial cell started with Aaron Novick. He gave me the appreciation of the experiments that he (with Leo Szilard) had done by simple bioassay to study the uptake kinetics of tryptophan. The experiments demonstrated that uptake was approximately hyperbolic even for a substrate needed in very small amounts by the cell. By using this novel approach they demonstrated that when a chemostat culture of a tryptophan auxotroph was limited by tryptophan, a mutant would arise and be selected because it had a lower K_m for tryptophan uptake than the parental culture had (81, 82). Later, I worked at the Institut Pasteur with Adam Kepes and Jacques Monod. Monod's group had detected and understood that membranes contained proteins that could function as concentrative pumps (80). This experience also led me to appreciate the subtleties of the galactoside permease. But in both of these intellectual milieux, I learned to appreciate the value of continuous culture and its use in modeling evolution and studying growth when conditions were poor. I was very greatly helped by Bill Baldwin, Doug Kell, and David White in constructing this review.

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AUTHOR'S CORRECTION

Microbial Physiology and Ecology of Slow Growth

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Volume 61, no. 3, p. 305–318: Because the issues of cells regulating their levels of enzymes were left for a subsequent review, key work from the laboratory of Ferenci (T. Ferenci, FEMS Microbiol. Rev. **18:**301–317, 1996; L. Notley-McRobb, A. Death, and T. Ferenci, Microbiology **143:**1909–1918, 1997) was omitted.

Page 310, column 2, lines 21 to 6 from the bottom: A two-component system regulating uptake and growth has already been described (J. W. Lengeler, Antonie Leeuwenhoek **63:**275–288, 1993).

Page 311, column 1, lines 10 to 13: Reference 75 discusses a straightforward kinetic model; the cybernetic model is discussed by the same authors in Chem. Eng. Sci. **52:**2567–2578, 1997.