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Bacteria in Their Natural Environments

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Koch) deals with the economics of bacterial growth in various nutrient regimes, and the second (by E. A. Dawes) concentrates on the survival of bacteria in starvation conditions and the role of storage compounds in survival. The next two chapters deal with aspects of bacterial survival in soil (by S. T. Williams) and aquatic (by R. Y. Morita) systems. Chapter 5 (by P. Morgan and C. S. Dow) is concerned with the prosthecate bacteria and their adaptations to low-nutrient environments. The final chapter (by R. H. Dainty) provides a contrast, as it deals with microbes in a nutrient-rich environment and thus allows a comparison of the problems encountered by microorganisms in such different nutrient conditions. Clearly, this volume does not provide a complete overview of all the features relevant to bacterial responses to environmental nutrient conditions—nor does it intend to. However, we hope that it highlights some important or new aspects of bacterial growth and survival and provides some appreciation of the flexibility and persistence of microorganisms.

We wish to thank the authors both for their excellent presentations at the Symposium and for their manuscripts. Our thanks also go to the Society for General Microbiology for mounting the meeting which served as the basis for this volume.

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The Macroeconomics of Bacterial Growth*

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Introduction

If every microorganism in the world needed to adapt only to a single constant environment, then 'Bergey's Manual of Determinative Bacteriology' (Buchanan and Gibbons, 1974) would be a lot simpler. And if the world ecosystem consisted of a series of permanent chemostat or turbidostat cultures, then the biology of microorganisms would be a lot less complex. These are the conclusions from the Darwinian dictum that 'the fittest survive,' which leads to organisms specifically adapted to their particular habitat, and from the Gaussian principle of 'competitive exclusion,' which leads to the elimination of species, thus reducing the complexity of ecosystems. In general, each long-term, continuous culture would have its resident organism well adapted to handling the particular problems of that environment. Few of these ecosystems would be diverse, and only a few would have multiple trophic levels.

The real world supports a much broader biota because of temporal fluctuations in physical factors and metabolic resources (Koch, 1974b,c; see Ricklefs, 1979; May, 1981), because alternate and multiple resources are sometimes available and because intermittant interconnections occur between habitats. No doubt this complexity was essential to the evolution of species, and the idealized world utopia for microorganisms pictured above, had it existed from the beginning, would have left many habitats unfilled, because organisms able to occupy many niches would never have had a chance to arise.

Consequently, continuous cultures of single organisms fail to serve as models for real conditions in nature, either past or present, but they can

* Dedicated to Hanna and Gerhard Colm.

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help us think about aspects of the problems. Moreover, the examination of bacterial growth under such idealized conditions is worthwhile in order to study microbial physiology, to select mutant organisms, to improve growth and yields and to study some special aspects of biology that have relevance to higher organisms. The purposes relative to this volume have to do with how bacteria attempt to cope with very little and how they cope in the presence of much.

One major pattern of microbial growth in nature is that new habitats arise and become inoculated with opportunistic organisms. They grow, enjoy an expanding economy, exploit the environment and occupy it rapidly, but eventually they alter that environment so that growth ceases and the population becomes static, declines or is replaced. In the other major growth pattern, a long-lasting habitat supports a standing crop of microorganisms. Growth does not cease, but rather continues at a slower rate, limited by new resources which enter the habitat either from the outside or from production by other organisms within the ecosystem. It is an un-proved but very likely assumption that a large fraction of growth of many kinds of microbes actually takes place in such nutrient-limited environments. Certainly, many organisms are adapted to coping with very low ambient concentrations of nutrients. This kind of growth situation applies when we consider both the oligotrophic environments of a freshwater lake or the gastrointestinal track of an animal. In both cases, for different reasons, the situation is one of low, but more or less continuing, supplies of nutrients.

Only when we study and understand the resultant problems and the concomitant adaptations will we be able to study intelligently the adaptations that allow the organism to cope with a fluctuating diverse environment. For the autecology of most microorganisms in nature, we need to understand how they respond under five kinds of circumstances. These are (1) total starvation of one or more needed nutrients; (2) chronic starvation as in chemostat culture; (3) good growth conditions as in batch or turbidostat culture; (4) the shifts between the first three states; and (5) the finding of new habitats. We microbiologists will do better in understanding the real world if we consider first the simplest growth situations and then proceed to the interplay of these. This chapter will ignore (1) and (5) but will consider (2), (3), and some aspects of (4).

Quantitative Growth

In this section the mathematics of growth is reconsidered; in part, this is done because much older thought on the subject has become embedded in

modern ecology (see any ecology text: for example, Ricklefs, 1979; Emlen, 1984) and microbiology (Monod, 1949; Painter and Marr, 1968) and, in part, because there are some new developments. Consider the case of an organism, reproducing by binary fission, which has constant properties as the result of extended growth in a well-mixed, uniform environment in which *either* no nutrient is critical *or* only the concentration of one nutrient is critical. In many cases, as the growth rate changes there are dramatic changes in cell size, significant changes in the relative proportions of macromolecules (see Maaløe and Kjeldgaard, 1966; Maaløe, 1979), but almost no change in the dry weight per genome. Therefore, if we choose to use the dry weight of bacteria per unit volume of culture, we will obtain results which are more generally applicable. Designating W as the dry weight of organisms per unit volume and S as the concentration of the relevant nutrient, the general form of the growth laws for batch or for chemostat growth are:

	Batch		Chemostat	
	$dW/dt = rW$	*or*	$[r - D]W$	(1)
and				
	$dS/dt = -rW/Y$	*or*	$[C - S]D - rW/Y$	(2)

In these equations r is the specific growth rate and is a function of the concentration of the critical nutrient. This is an important consideration and much of discussion below depends on the shape of the functional dependence of r on S. Y is the yield of cells from a unit quantity of this nutrient. The form of the equations on the right refers to chemostat culture, and D is the dilution rate (equal to the flow rate divided by the culture volume). C is the concentration of the resource in the reservoir (input) medium and also the concentration in the growth chamber when inoculated at time zero into chemostat or batch growth.

Mathematically, when combined with expressions for r as a function S, these systems of differential equations can be intractible and may not yield an exact solution applicable to the actual circumstances of real cultures. This is because of the way in which W and S appear in the equations and because r actually is a complicated function of S. But of course there are very important limiting cases. If S is large and constant, growth is exponential and given by W_0e^{rt} or $W_0e^{(r-D)t}$, where W_0 is the initial dry weight of organisms per unit volume and t is time. If S is zero, then r is zero (unless the cells have utilizable reserves), and growth does not take place; but wash-out, if possible, will occur. Under chemostat conditions, a steady state is achieved where dW/dt and dS/dt are both

zero, and then $r = D$ and $(C - S)\,Y = W$. Under batch growth, if C was the concentration at a time when the inoculum was W_0, and growth was followed until $S = 0$, then $CY = W - W_0$. The idealization of these formulations neglects the effects of new mutants, adaptations, the possibility that r might depend on W as well as S, physiological changes during the stationary state, wall growth and death. Still, it applies quite frequently and well.

Historically, Verhulst (1838) made the assumption that organisms converted resources into themselves in a special autocatalytic manner. To grasp this basic thinking, consider a case where his logic would work: trypsin recruiting more trypsin by activating trypsinogen. In this case, r is directly proportional to S, and the initial rate would be proportional to the initial concentration C. The rate of growth at later stages would depend on the residual resource (trypsinogen concentration). In our symbols the amount yet to be created is $CY + W_0 - W$ or $K - W$, where K is the so-called carrying capacity. The specific growth rate for this case is simply $r = r_{max}(K - W)$. For batch growth, Eq. (2) becomes superfluous because we have now taken it into account, and Eq. (1) becomes the logistic differential equation:

$$dW/dt = r_{max}(K - W)W \quad (3)$$

The logistic is famous in part because it can be solved, but in larger part because it gave rise to the concepts that growth had two aspects: r and K. Ecologists consider that certain organisms have adopted evolutionary and ecological strategies that allow them to increase r to larger values than those of potential competitors; these are r-strategists and are the rapid growers. Alternatively, they may have evolved mechanisms to increase K; these are the K-strategists and are organisms that are better competitors in a crowded situation. The reader will recognize that it is a far cry from trypsinogen activation to ecosystems of higher plants, animals and microbes, but the ideas have been carried over and form the cornerstone of quantitative and qualitative ecology.

However, the logistic case is not the appropriate model for most cases of bacterial growth. This is because it is only rarely that the rate at which bacteria can find and utilize a resource is directly proportional to the resource's concentration. This applies when the resource concentration is very small and diffusion limits the organism's ability to capture the nutrient (see below). It also would be appropriate when the uptake system of the bacteria is not avid, that is, when the uptake system is very inefficient. Then doubling the concentration would double the chances of collection and hence the rate of uptake. Only in very special circumstances is this latter case relevant, because uptake systems are such an important part of

the bacterium's economy that they have, in most instances, been subject to strong evolutionary selection.

There are three contending choices for the dependence of r on S for microbiological problems. First is the "Blackman law of the minimum" (Blackman, 1905), which assumes that below a critical concentration, growth is directly proportional to S, and above this concentration growth is independent of S:

$$r = a_1 S; \quad S \leq r_{max}/a_1$$
$$r = r_{max}; \quad S > r_{max}/a_1 \quad (4)$$

where a_1 is the second-order rate constant.

This model was developed by a plant physiologist at the turn of the century and is based on earlier ideas of Justus Liebig in 1843 and introduced into microbiology by Koch (1971, 1972) and Dabes *et al.* (1973). It would apply if the uptake capacity at high S greatly exceeded the maximum growth rate limited by other causes.

A more popular expression is that of Monod (1942), which was derived by analogy to enzyme kinetics. It is

$$r = r_{max}S/(K_m + S) \quad (5)$$

where K_m is the Michaelis constant. This relationship has been generally used in chemostat theory, fermenter operations, microbial ecology and microbial physiology.

The more general expression has been derived by Best (1955) and rederived and exploited by Powell (1967), Koch and Coffman (1970), Dabes *et al.* (1973) and Zimmermann and Rosselet (1977) for microbiological problems. I have recently shown that this formulation applies to glucose utilization by *Escherichia coli* (Koch and Wang, 1982; Koch, 1982a,b) better than the other two [Eqs. (4) and (5)]. It is

$$r = r_{max}\,(S + K_m + J)\{1 - [1 - 4SJ/(S + K_m + J)^2]^{1/2}\}/2J \quad (6)$$

In this expression $J = r_{max}/(AP)$, where A is the surface area of the bacteria and P is the permeability constant, that is, the diffusion constant divided by the thickness of the membrane; K_m is the half-saturating concentration for diffusion. It applies when transport into the cell involves two steps: a passive diffusion process and a subsequent step that exhibits irreversible enzyme kinetics. For Gram-negative bacteria, these two steps are found to be associated with diffusion through the porins in the outer membrane and with the permease or other uptake through the transport system across the cytoplasmic membrane. Figure 1 shows the gradation in response to substrate concentration as the parameter J changes. The

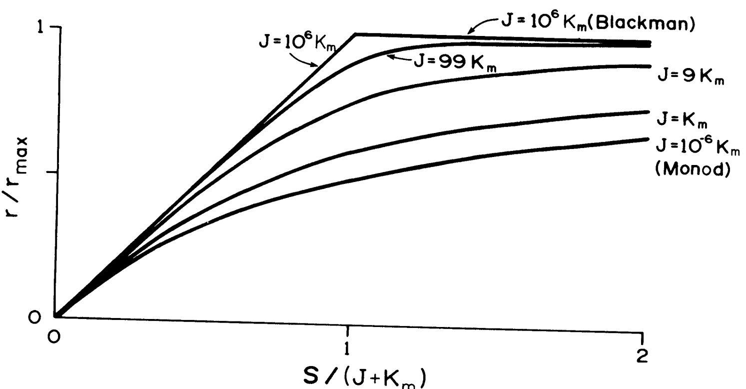

Fig. 1. Theoretical dependence of growth rate on substrate concentration. The abscissa represents the substrate concentration measured in multiples of the sum of the growth parameters, J and K_m (see text). The relative growth rates were calculated from Eq. (6). The limiting values of $J \rightarrow O$ lead to the Monod model (hyperbolic dependency), and the limit $J \rightarrow \infty$ approaches the Blackman model with two straight-line segments.

general Best model approximates to the Monod expression when J is small and to the Blackman expression when J is large. For the growth situation that we have studied (Koch and Wang, 1982), the value of J is such that the response is more nearly like the Blackman model.

Returning now to Eqs. (1) and (2), we are now in a position to calculate batch growth curves for each of the cases. For the Blackman case, growth is pieced together from

$$W = W_0 e^{(r_{max})t}, \tag{7}$$

for the portion where growth is independent of S, and the integral form of the logistic equations where the growth rate is directly proportional to S is

$$W = W_c\, e^{[r'(t-t_c)]}/[1 - W_c/W_{max} + W_c/W_{max} e^{[r'(t-t_c)]}] \tag{8}$$

W_{max} is the maximal titer [$= CY$ for batch growth], W_c and t_c are the critical dry weight and time, respectively, at which S has just decreased enough to become growth limiting and $r' = r_{max} W_{max}/(W_{max} - W_e)$. For the Monod case, the differential equation is also soluble, and Monod (1942) himself provided the solution:

$$tr_{max} = \frac{K + C + W_0/Y}{C + W_0/Y} \ln[W/W_0] - \frac{K_m}{C + W_0/Y} \ln[(C + W_0/Y - W/Y)/C] \tag{9}$$

No analytical solution is possible for the more general case utilizing Eq. (6), but numerical methods can be used.

Although numerical methods can be applied, they cannot be easily or simply used, because Eqs. (1) and (2) together with either (4), (5) or (6) define a 'stiff' system of differential equations. This means that when a time simulation on a computer is set up, it may proceed accurately, calculating the values of W and S after successive intervals of time. All may go well for a while, but then suddenly, when S falls too low, the computer extrapolation fails to give a valid answer, as indicated by the output of negative and erratic values. The computer routine may partially ameliorate this situation by using smaller time intervals, but in many cases such small time intervals are needed that the calculation becomes too expensive. The general treatment of such stiff systems is the subject of intense study by numerical analysts, and very sophisticated software packages are available (Gear, 1981). However, for the curves presented in this section, I wrote a program for a pocket programmable computer that was able to handle the problems with only the use of a fourth-order Kutta-Runge procedure and careful control of step size by the operator and the software program. For the application used here, the assumption of material balance (that is, $dW/dt + Y\, dS/dt = 0$) was appropriate, because batch or chemostat growth with one limiting substance was being considered.

But why bother? Under most culture conditions in which a single nutrient may be critical, one of the three limiting expressions (for either exponential growth, or steady-state chemostat growth or no growth at all) will usually apply. It is only at the transitions between phases of growth that the much more complex calculations are needed. However, I thought that carrying out this kind of computation was worth the work to better understand growth under oligotrophic conditions and chemostat growth as fast dilution rates (approaching wash-out). In future work, these programs should aid in studies of shifts between growth modes, as mentioned above, and of instabilities that show up in certain cases of continuous culture.

Figure 2 shows theoretical growth curves for the Monod case of chemostat growth that are quite good representations of actual experimental curves. When K_m/C is small, growth follows the exponential curve, and then the increase in biomass concentration abruptly stops. This applies when the only carbon source is an avidly scavaged substance such as glucose at a low concentration. For batch growth under these conditions, we say 'growth stopped in exponential growth' (see Koch, 1971). To achieve a less abrupt leveling off of growth, either K_m must be increased or C must decrease. To change K_m, usually a different limiting growth factor must be used; to decrease C means that the final value of W must be smaller and more sensitive measurements of it must be made. Viable

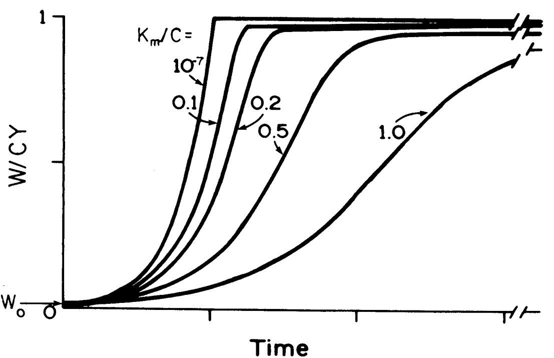

Fig. 2. Approach to chemostat steady state for cell growth following the Monod model. The curves are time simulations, calculated as indicated in the text. Because the system of differential equations is 'stiff,' special numerical procedures are needed, especially where the curves appear to have a discontinuity, particularly in regions where K_m/C is small.

counts can be made which are very sensitive, and total counts with an electronic particle counter can be almost as sensitive (see Koch, 1981b). However, we have used a long path cuvette (10 cm) in a double-beam spectrophotometer (Wang and Koch, 1978) connected to a computer. In this way, very precise growth curves were obtained (Koch and Wang, 1982). To distinguish between Eqs. (4), (5) and (6), and a number of other possibilities, growth to completion on a very low level of glucose was followed. My on-line computer calculated the specific growth rate from 300 successive turbidity measurements obtained during a 200 s interval. The logarithm of the corrected turbidity was regressed against time to obtain an r value. The substrate concentration was computed from the growth yield and the regression estimate of W at the start of the interval. Then a large number of these data pairs was used by another computer to construct a representation of the growth, as in Fig. 3a, and to calculate non-linear statistical fits to the three models mentioned above and to other models (Koch, 1982a,b). The Best model made the closest fit within the constraint of choosing a saturable growth rate function.

But it is the growth curves themselves and not the r vs S relationship that are of interest in this section, and Fig. 3b shows the growth curve from the same set of data (Koch and Wang, 1982; Koch, 1982a,b). Superimposed on the data points ($\bar{W}$, t) are the growth curves calculated for the best fitted parameters of the three models. Even when initial carbon source concentration was 0.0005% glucose, it can be seen that in the integral format the decision between growth models is not as clear-cut as with the r/S representation of the data (Fig. 3a). In either case, the best fitting model for this experimental situation is still the Best model. It and

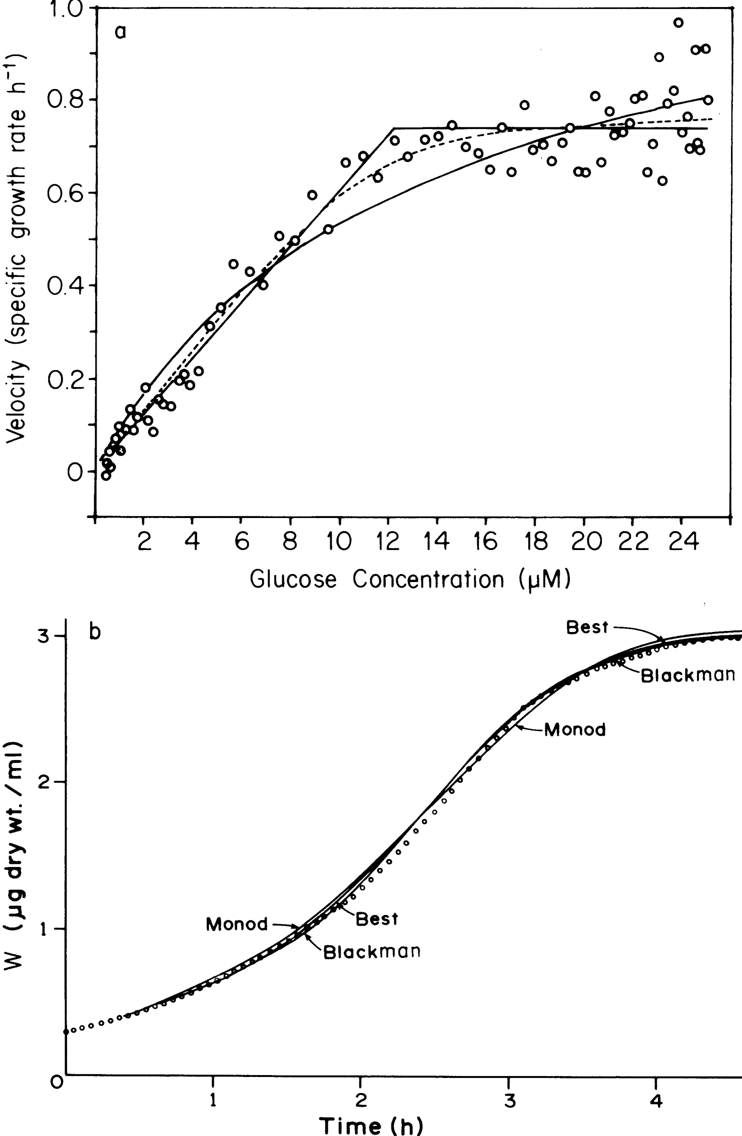

Fig. 3. Fit of various growth models to experimental data. Each point represents the growth measured over a 200 s interval by a computer-linked absolute turbidometer (see text). The set of points show the deceleration of growth in very low glucose of *Escherichia coli* stain ML308 as the substrate concentration and the growth rate fell to zero. (a) Rate plot in which the data on graphed analogously to Fig. 1. The curves are non-linear least-square fits for Monod and Blackman models (solid lines) and Best model (dashed line). After Koch and Wang (1982). (b) Growth curve representation of the same data. The curves are computed from the best fitting parameters derived from (a) to reconstruct the growth curves. The choice between the models based on the experimental data is not as clear as in the data representation of (a).

the Blackman provide better fits than the Monod model. While under almost laboratory conditions the difference between the models would be even more insignificant than those shown in Fig. 3b, when the available nutrient is low as it is in many natural oligotrophic environments, the differences in the growth curves and their interpretation will become increasingly important.

Chemostat Growth Near the Wash-out Rate

During steady-state chemostat operation, the left-hand sides of Eqs. (1) and (2) will be zero, and one can solve for the steady-state level of bacteria predicted by each model. Figure 4 shows the steady-state standing crop as a function of dilution rate for the models of Monod and Blackman. As has been noted before (see Herbert, 1958), the balance of growth rate and wash-out rate shifts the steady-state value of W more or less abruptly with D; the break at low K_m is almost discontinuous for the Monod case. It is fully discontinuous for the Blackman case (thin line, Fig. 4). Therefore, it is intermediary in character for the Best model for J values between 0 and infinity. This can be seen in Fig. 5 which stimulates the steady-state behavior for the growth responses of *Escherichia coli* shown in Fig. 3. This means chemostat growth at D approaching r_{max} could be quite unstable and sensitive to minor fluctuations in pump speed and

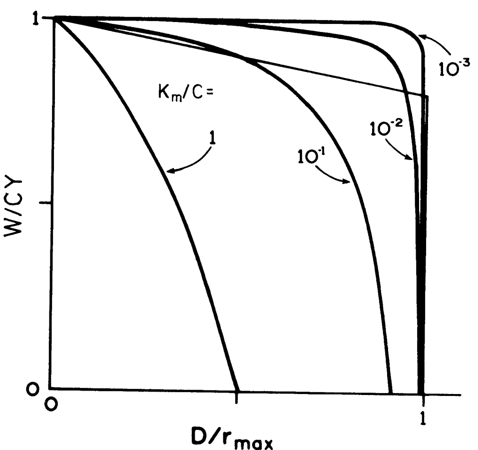

Fig. 4. Standing crop during chemostat growth. The standing crop (W/CY) is shown for various dilution rates (D/r_{max}) for various values of K_m/C for the Monod model and one case for the Blackman model (see text).

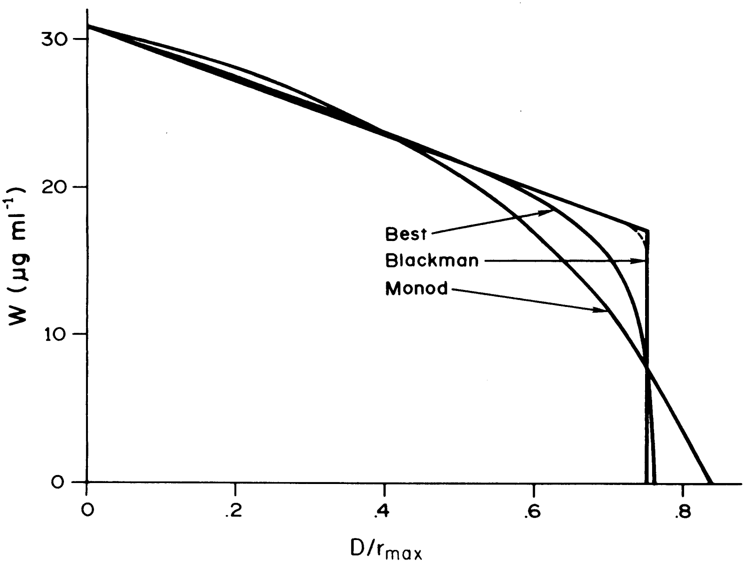

Fig. 5. Standing crop for chemostat culture of *Escherichia coli*. The best fitting parameters for the three models were used to predict the standing crop of bacteria (W) under chemostat growth of ML308 with $C = 0.0005\%$ glucose at various dilution rates (D/r_{max}).

uniformity of stirring; studies of this situation will be presented in a later publication.

Even at slower growth rates (lower ratios of D/r_{max}) the value of J is important. Figure 6 shows the approach to steady-state of chemostat cultures inoculated with 1% of the biomass that could be formed from the amount of limiting substrate. All curves correspond to a constant value of K_m + J, but differ in the partition between K_m and J and in the dilution rate. These curves should be characteristic of growth in many oligotrophic situations and provide a method of analysis of experimental studies.

Life at Low Substrate

In this section I wish to address the problem of coping with chronically too little, but I will not consider topics covered in the recent reviews of Harder and Dijkhuizen (1983). The experimental paradigm is a nutrient-limited continuous culture chemostat or cultures supporting very sparse growth that then must be studied with special techniques, such as radioac-

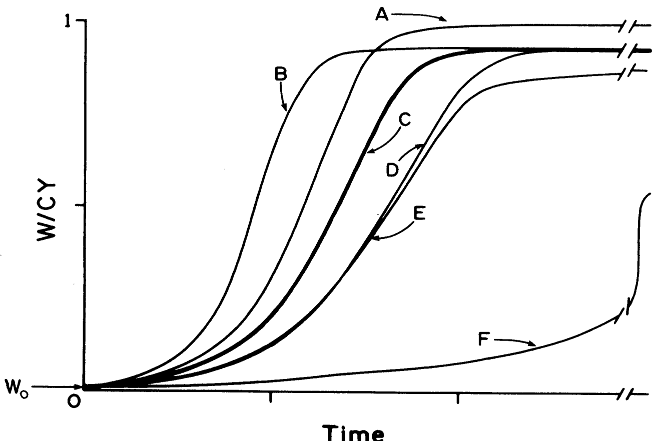

Fig. 6. Growth under continuous dilution for the Best model. Growth curves from an inoculum W_o of 1% of the maximum yield based on the inflow to a chemostat culture. The following parameters were adopted.

Curve	D	K_m	J
A	0.1	30	30
B	1	1	59
C	1	30	30
D	1	59	1
E	2	30	30
F	5	30	30

Curve C is drawn more heavily and the other curves are variations.

tivity uptake, particle or colony counts or long-path turbidity. For nutrients that are of small molecular weight, even in a well-stirred vessel, diffusion at one or more of the five regions shown in Fig. 7 can be critical in controlling the flow of nutrients in reaching the metabolic site within the microorganism in order to be utilized. In its simplest form, Fick's diffusion law can be expressed:

$$dq/dt + DA\left(\frac{dC}{dx}\right) \tag{10}$$

and relates the quantity transported per unit time, dq/dt, to the concentration gradient, dC/dx. The proportionality factor is made up of the product of the area, A, across which the diffusion takes place and the diffusion constant, D.

Surface-to-volume Ratio

When transport in regions II, III or IV (Fig. 7) is the growth-limiting factor, a Gram-negative organism can cope only by altering the things under its control; for example, the outer membrane could be made thinner with more open or wider porin pores, the periplasmic space made smaller or active transport systems made more numerous or efficient. There is a general recourse: the cell may alter its size adaptively or genetically. Basically, microbes are small because of the need for a sufficiently large value of dq/dt, so that a given volume of protoplasm is supplied with nutrients fast enough to survive and grow. Because the volume increases as the cube, but the area only as the square of the linear dimension, a sufficiently large value of dq/dt for a given unit volume of protoplasm can, in principle, always be obtained by alteration of the microorganism's size to a sufficiently small value without any other physiological changes. Therefore, microorganisms are 'micro'; seemingly, they should be as small as possible conditional on other constraints. In fact, *Escherichia coli* is smaller when growing at a slower dilution rate in chemostat culture.

Let us consider the various microbial strategies to achieve the high surface-to-volume ratio. As noted, we must focus on the packaging of a unit volume of protoplasm. An inherently bad plan for doing this is the

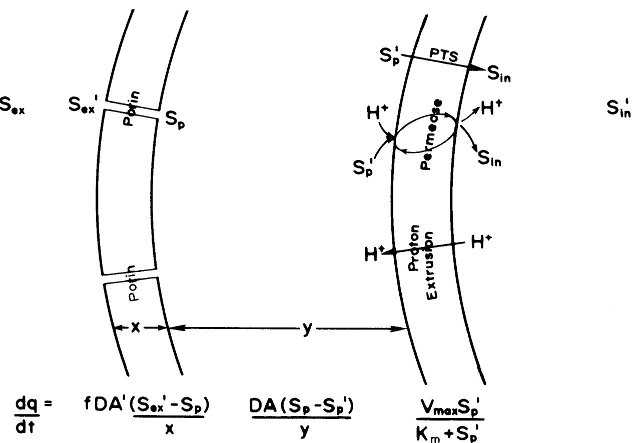

Fig. 7. Regions in which diffusion may limit growth (see text). The expressions listed below regions II, III and IV indicate the relevant diffusion or transport equation.

strategy of the cocci. With this basic morphology, becoming smaller is the only way to improve the surface-to-volume ratio. It is a bad strategy because a sphere has the poorest surface-to-volume ratio of any possible solid. Nevertheless, the diplococcus and budding yeast approaches this shape quite closely. The optimum strategy, if restricted to simple geometric shapes, is to become a very long thin filament or better to become flattened like a thin leaf. In the latter conformations, the maximal surface-to-volume ratio could be achieved. As far as I am aware, no single-celled microbe, except for the recently discovered square bacteria of Walsby (1980), utilizes the latter strategy. It is not yet understood how such a shape develops or how it avoids having an osmotic pressure that would result in a tendency to swell into a more spherical shape. Therefore, at present, no speculation as to why this shape is not more common or why other microorganisms have not adopted it can be presented.

A better approach than the diplococcus strategy is that of adopting a rod-shaped habit of growth. A rod-shaped organism with hemispherical poles has a surface area, A_r, relative to the area of a spherical cell, A_s, of the same volume given by

$$A_r/A_s = \varepsilon[4/(6\varepsilon - 2]^{2/3} \qquad (11)$$

In this expression, ϵ is the ratio of length to width of the rod-shaped cell. From the values of A_r/A_s as given in Table 1, it can be seen that the eccentricity (ϵ) of the cell must become very large to make major changes is the area available for absorption of nutrients. Inasmuch as typical rod-shaped organisms have a birth size two times longer than their width, ϵ varies from 2 to 4. On the average it is closer to 2 because the small cells are more numerous than the large cells in a growing population. It can be seen that there is only very little improvement (~10%) in surface-to-volume ratio for this degree of asymmetry. This implies one of two things: either contrary to conventional wisdom, rod-shaped morphology does not serve as an important improvement as far as uptake of nutrients is concerned but is so commonly found for some other reason, or indeed, this aspect is so extremely important for bacterial growth that even an increase of a few percent in surface area per unit volume of cytoplasm will greatly increase the selective advantage of the organisms in nature. But then why are organisms not even longer and thinner? Therefore, the second possibility appears less likely unless some other quite general reason as to why cells cannot be made thinner and longer is found. Possibly there are mechanical problems involved in cylindrical growth process (see Koch, 1983a).

Table 1. *The ratio of the surface area of a rod-shaped cell (Ar) to that of the equivalent sphere (As)*

Eccentricity[a] ε	Ratio A_r/A_s	Eccentricity ε	Ratio A_r/A_s
1.0	1	10	1.682
2	1.086	20	2.095
3	1.191	30	2.389
4	1.284	40	2.625
5	1.366	50	2.824
6	1.441	60	2.999
7	1.570	70	3.155
8	1.598	80	3.298
9	1.628	90	3.4285

[a] Eccentricity is the ratio of length to width of the organism.

A similar calculation can be made for the appendages, that occur on *Hyphomicrobium, Caulobacter,* and other prosthecate bacteria, that are tubular extentions of the peptidoglycan wall with presumably the same overlying and underlying membranes that are present in the rest of the cell wall of these organisms. If such cells are idealized as being spherical with a cylindrical tube projecting from them, the surface for a fixed total cytoplasmic volume for the cells can be calculated. The relative surface area, A_r/A_s, as a function of radius and length of the appendage is shown in Fig. 8. The conclusion is that a significant increase in surface area by these appendages could be extremely important to the bacteria, as suggested by Pate and Ordal (1965) and Poindexter (1981).

Diffusion through the Environment

The considerations so far have been based on the concept that surface area per unit volume is the important design factor for microorganisms. This concept applies whether or not transport through regions II, III or IV of Fig. 7 is critical. It applies whether or not transport depends on passive diffusion, consistent with Fick's law of diffusion, or occurs by a more complicated facilitated diffusion process or by an active transport mechanism. In all three the rate is proportional to the surface area.

However, there is another aspect of nutrient uptake which dictates some of the same conclusions and is critically important to oligotrophs. This is the problem of diffusion of nutrients through the environment up to microorganisms, that is, through region I of Fig. 7. If the external concentration is very low and the cell uptake mechanisms are capable of

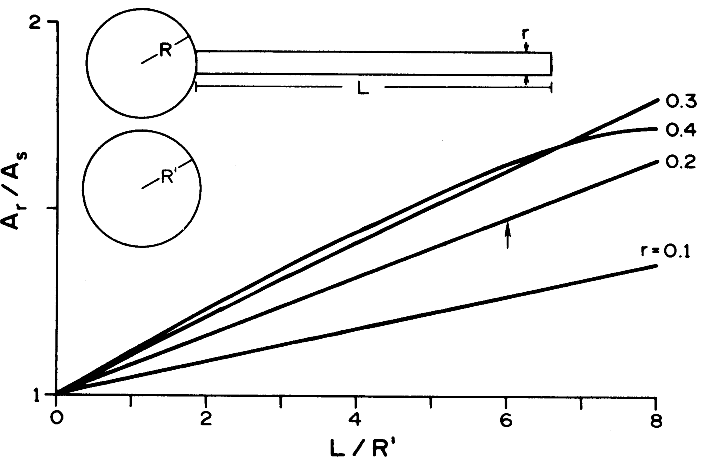

Fig. 8. Relative surface area of appendaged organisms. A_r/A_s is the surface area of an appendaged cell relative to a spherical cell of the same volume. The abscissa is the ratio of the length of the appendage to the radius of the equivalent sphere (L/R'). The different curves are marked with the value of r as a fraction of R'.

lowering the concentration at the surface to near zero, then diffusion through the surrounding medium can become the rate-limiting process. In this case, a different body of mathematics is needed, and the geometrical relationships are different. This was originally formulated by von Smoluchowski (1915, 1918). The derivation starts by rearranging Fick's law in the form of a differential equation suitable for a spherical object. Then, by assuming that the cell is capable of maintaining the concentration at its surface at zero, while the bulk solution concentration at large distances remains at some original fixed value, this leads to a steady-state equation:

$$dq/dt = 4\pi DRC \tag{12}$$

where R is the radius of the cell and C is the bulk concentration. This was recast into a form relevent to microbial growth (Koch, 1971), which states that the maximal efficiency with which a spherical stationary microorganism could clear its environment by uptake of nutrient molecules bombarding it by Brownian motion is equal to $3D/R^2$. Efficiency in this case is defined as the number of equivalent cell volumes of medium that the cell would clear of nutrient simply by absorbing all molecules that collided with it through diffusion. Consequently, for an oligotrophic environment a smaller radius improves the efficiency in the same qualitative manner, but not in quite the same quantitative way as when the limitation is within the cell envelope. Consequently, this is the second aspect of diffusion that

forces microorganisms to be as small as they possibly can. As a numerical example, a spherical cell of the same volume as a typical strain of *Escherichia coli* growing at 37°C in minimal medium could clear a maximum of 2800 cellular volumes per second. This upper limit could, in principle, be further increased by very strong stirring or by very high motility, but neither shear forces in a laboratory fermenter nor motility in the normal range (Koch, 1971) will appreciably aid well-dispersed microorganisms in harvesting a nutrient present in very low levels in their medium. This same point was made later by Berg and Purcell (1977) (see also Berg, 1975; Purcell, 1977).

Bacterial uptake systems do become so effective that uptake is restricted by diffusion. The copiotroph, *Escherichia coli,* when adapted to glucose-limited chemostat conditions, was found to have its efficiency raised from 72 to 651 s^{-1} (Koch and Wang, 1982). Moreover, most of the limitation developed in region II (Fig. 7), as deduced from value of J obtained as indicated above. This implied that without its outer membrane, *Escherichia coli* would have had an efficiency 3280 s^{-1}, to be compared with the theoretical value of $3D/R^2$ for these conditions of 3700 s^{-1}. It is to be noted that this copiotroph was more efficient than two oligotrophs studied earlier by Matin and Veldkamp (1978). I hope that measurement of efficiency becomes a more routine part of microbial ecology and that a sufficiently high value, approaching $3D/R^2$, becomes a criterion for true oligotrophs.

The expression of maximal efficiency, $3D/R^2$, applies nominally only to spherical cells. Approximate calculations (Koch, 1971) showed that long, rod-shaped or filamentous cells need to remain in motion with respect to their fluid environment to continue to take up nutrient at a maximal rate. An extension of these calculations (A. L. Koch, unpublished) shows that short rods can be well approximated by the treatment of spheres with the same volume, but the mathematical problems so far have prevented a critical analysis. Still, it can be asserted that a stalk or other appendages could greatly improve the ability of the cell to scavenge the external medium. This corresponds to the old observation that a leaf with only a minute fraction of its surface exposed through open stomata can transpire water at nearly the same rate as wetted piece of blotting paper of the same dimensions.

This is only one of several pertinent geometric situations in which diffusion operates differently than intuition might suggest. A case involving virus adsorption illustrates the same problems associated with the adsorption of certain nutrients. It had been wrongly assumed (Puck *et al.*, 1951) that because the von Smoluchowski relationship [Eq. (12)] held for adsorption of coliphage by bacteria, this implied that every collision was

fruitful. However, a combination of collision theory and the diffusion theory showed that only a small fraction of the collisons need be fruitful (Koch, 1960). A different approach which came to the same conclusion was presented Berg and Purcell (1977). The latter paper derived an exact expression for the fraction of the surface that must be absorptive for efficient uptake by the whole spherical cell. This is simply Eq. (12) multiplied by $Ns/(\pi a + Ns)$, where s is the radius of the absorptive site, a is the radius of the cell and N is the number of sites per cell. For example, with $s = 50$ nm and $a = 1$ μm, 90% of the absorptive rate would be achieved with $N = 565$ receptor sites per cell covering only 35% of the surface; 50% of the absorptive rate would require 63 receptor sites covering 16% of the surface.

Even fewer sites would do on the cytoplasmic membrane if the molecules were contained by the outer membrane, as in the case of Gram-negative organisms. Moreover, if there were agents (binding proteins?) that could bind the solute, loosely bind to the membrane and diffuse to the receptor adhering to the membrane, then a two-stage capture (Adams and Delbruck, 1968) could be even more effective.

Why Can't a Cell Grow Infinitely Fast?

Steady-state growth where r is constant is established when cells are maintained under constant conditions. While r depends on assimilation when an essential component is limiting, under conditions where no component is limiting, the kinetics depend on intracellular processes. The metabolic processes carried out by an organism are myriad. Most of the products of individual steps in a cell in a growing, expanding economy act as positive feedback to increase production of their own producer. This causes the growth kinetics in terms of the rates of intracellular processes to be surprisingly simple according to Hinshelwood (1946). It is even simpler than he surmised, however, not for a biochemical reason but for an economic one, resulting from the fact that most of the cell's activity is directed toward making protein. Thus, ribosomes and associated proteins make proteins that, in turn, make, or serve an indirect role in making, the ribosomes and associated factors that made them, leading to an expanding spiral. In broad strokes we can represent the growth rate controlling steps of cell metabolism as

(13)

where R is the concentration of the complex of proteins and RNA involved in the production of the ensemble of cellular proteins, of concentration P. The pseudo-first-order rate constants for the two overall processes are designated k and c. They can be experimentally measured in a balanced growing culture from $k = (dR/dt)/P$ and $c = (dP/dt)/R$. It has been argued (Koch and Schaechter, 1962; Koch, 1970, 1971, 1976, 1979) that in a growing organism in the steady state the production of other cell constituents, morphological entities and other cell processes are forced to tag along. Thus, such diverse processes as chromosome replication, flagellum formation, construction of apparatus for energy production, formation of a pre-cell division constriction and other peripheral metabolic procedures are passively regulated, if not regulated in a more sophisticated way through the production of proteins and/or protein-synthesizing machinery.

Backed up by physiological data, the economic argument is that it behooves the bacterium to regulate its business so that lack of minor or cheap items does not prevent growth when the ēxpensive and major materials are available.

In any case during balanced growth under constant conditions, no matter how sophisticated or unsophisticated (passive) the regulatory system of the cell, all cellular components will increase with the same specific growth rate given mathematically by

$$r = \sqrt{kc} \tag{14}$$

Given unlimited resources, a cell culture would increase exponentially, and hypothetically the rate of protoplasm formation per culture would increase indefinitely. However, the rate of cell production per cell remains constant because cell division is also linked to cell growth. Note that it does not matter if cell division regularly produces large or small cells; either way, as long as k and c stay constant, r stays constant. But the primary question is 'What sets and limits the specific growth rate constant, r?'

What sets the values of k and c that make up r? Each of these is a lumped parameter: a pseudo-first-order rate constant made up of many rate constants. The value of the appropriate constituent constants in the mathematics of steady-state growth determines the ratio of any cellular constituent to any other one, and thus the kinetic properties of key enzymes and biochemical reactions in the long run must control the system and thus set r.

How can r be changed? Evidently, mutations that affect many kinetic properties of any cellular process could affect r. If the mutation affected a more main-line process controlling either k or c, the effect would be more

direct than if it were affected by some dependent peripheral process. In the latter case, mere quantitative changes might have no effect on the specific growth rate because pool sizes would change (see Harvey, 1978; Koch, 1981a). Concomitantly, even great changes in the rates of dependent processes would have only minor changes in growth rate, although complete stoppage would eventually prevent growth. Obviously, mutations in regulatory circuits would have effects that might not be apparent in particular circumstances. But to the degree that evolution has produced bacterial *r*-strategists, many wild-type organisms from nature should be capable of very fast growth rates if we provide the optimal environment.

Experimentally, we can add or subtract single specific nutrients or inhibitors, improve the complex of nutrients in the environment or alter the temperature. Variation of some of these parameters—such as temperature, the concentration of certain heavy metals, and the concentration of many organic components—make r pass through an optimum. So one answer to the original question is that since many things are both good and bad, the maximum growth rate is limited by the best compromise.

Another answer comes from the saturability of enzyme and transport systems. For most enzyme reactions the rate, v, can be pushed only to V_{max}. This could limit the growth rate if r was controlled by processes that cannot be speeded up by an increase in number of catalytic units. Thus, inherently, protein synthesis is limited because only one ribosome at a time can read a particular segment of message. Other processes with such a rate-limiting step determine how fast an RNA polymerase holoenzyme can copy DNA, how fast an rDNA gene can be initiated and replicated or how fast a holo DNA polymerase can copy DNA on the leading strand. In a growing cell these bottleneck limitations can be overcome to a degree by standard tricks such as (1) bidirection replication of DNA and multifork dichotomous replication of the chromosome, (2) multiple copies of rRNA genes and certain structural genes and (3) rapid reinitiation of transcript and translation. These tricks work to a limited degree before inherent disadvantages outweigh the advantages. For example, topological restraints may prevent too many DNA replication processes occurring simultaneously on the chromosome. Consequently, another answer to the original question is that the maximum growth rate is essentially limited by one or more of the cellular bottlenecks where only the opportune enzyme can function, and additional copies will not have a chance, or at least must wait their turn to function.

As additional substances are provided, an organism such as *Escherichia coli* needs less and less of its protein synthesis directed toward intermediary metabolism. Progressively, the problem becomes one of macromolecular synthesis, energetics and transport. By extrapolation,

the cell would be pure ribosomes if the doubling time were reduced to 10 min at 37°C (R. Harvey, personal communication). That is to say that a cell composed only of ribosomes and associated factors engaged in making only ribosomes and associated factors would have a 10-min doubling time. If ribosomes that could work as well but had one-fourth the weight could be constructed, then the maximum theoretical specific growth rate could be nearly 5 min.

Another concern is energy generation, because both macromolecular synthesis and transport events consume a great deal of energy. In fact, Stouthamer (1977, 1979) calculates that it takes a comparable amount of energy to transport amino acids, purines and pyrimidines as it does to make them from glucose and a nitrogen source. So it may be that energy generation in the forms of proton gradients, ATP and phosphoenolpyruvate production might limit the maximal cell growth rate.

How could these possibilities be tested? The critical way to find the rate-limiting step in a steady-state system is to have all the components on hand in purified form and add a small quantity of each, one at a time, to the system and expect that only the limiting component will augment the rate. In principle, reducing the level of each component one at a time would do as well. Pharmacological studies with inhibitors can be used in this way, but only if a concentration of the drug can be proven to remove effectively a specified portion of a single component from function in the steady-state system. Since these relationships are usually not well defined, this approach has seldom been used even though it is inherently very powerful.

This is the approach that we (Harvey and Koch, 1980; A. L. Koch and R. J. Harvey, unpublished) have taken. We have studied a strain of *Escherichia coli* ML30 which descended from a cell isolated from nature 50 years ago. This strain has not been subject to a series of mutagenic procedures as have most K-12 derivatives which may account for its faster growth. Its growth was studied in two media, one containing minimal salts plus glucose and the other containing those ingredients but enriched with tryptone and yeast extract. In this latter medium, under forced aeration, a doubling time at 37°C of 16 min (specific growth rate, 2.60 h^{-1} or 3.15 doublings h^{-1}) could regularly be produced (Koch, 1980). This is appreciably faster (25%) than the fastest growth rate reported in most physiological studies, which is a doubling time of 20 min. This 25% faster growth occurs if only one mainline reaction rate constant is affected to increase by 57%. The 16-min doubling time is not much slower than Harvey's 10-min autocatalytic ribosome.

In previous studies (Harvey and Koch, 1980) the quantitative effect of a partially inhibitory concentration of chloramphenicol (CAP) on growth

was measured. Briefly, it was found that the combined effect of CAP blocking protein synthesis and its effect on slowing the maturation of the ribosome RNA precursor predicted the observed measurement of specific growth rate according to $r = \sqrt{kc}$ [Eq. (14)] for partially inhibitory concentrations of CAP (0.5–6 μM).

These studies were done in the minimal-glucose medium at 30°C with aeration. Under these conditions the doubling time was 58 min. An extremely important finding was that there was no threshold in the action of this drug. Thus the degree of inhibition of r increased progressively with drug concentration from 0 μM CAP. The conclusion is that some step in protein synthesis or ribosomal RNA maturation is rate limiting for bacterial growth under these conditions. Since the 50% inhibition of protein synthesis occurred at 2 μM CAP and the inhibition of maturation of rRNA occurred at 2.8 μM CAP, the effect of the inhibition of the protein synthesis is greater than the effect of the inhibition of ribosome maturation. When a small fraction of the L16 of the 50 S ribosomal subunit binds CAP, the rate of global protein synthesis slows proportionately, and that inhibition of maturation of an rRNA also slows growth. That is not surprising, but it can be concluded that other cellular processes are not so limiting.

This result suggests that there are no excess ribosomes and probably no excess capacity to synthesize rRNA. This is reminiscent of the constant efficiency hypothesis (Maaløe and Kjeldgaard, 1966; Maaløe, 1979), which states that at a given growth condition the cell makes only enough ribosomes so that they may all be fully employed. To recap the argument set out above, this is economically optimal because the ribosomes are the expensive 'capital' equipment of the cell (Koch, 1971), and it would be expedient if the cell arranged its other activities to maximize the use of each ribosome. This argument has been presented quite often in the literature and is in rough accord with the facts over the central range of growth rates. However, it does not hold for very slow growth rates (Koch and Deppe, 1971; Alton and Koch, 1974) nor for very rapid growth rates (Koch, 1980). This means that under poor and even moderate growth conditions more ribosomes are made than are employed at any instant of time. These excess ribosomes are functional, and after a shift-up, they serve to form proteins in less time than it takes to produce new ribosomes. Presumably (Koch, 1971), this allows the organisms to respond quickly to an improvement in growth conditions. But clearly they are not recruited when some ribosomes are pulled out of action by CAP.

The rate of protein synthesis after the removal of the limitation of a slow growth rate in chemostat culture in defined minimal medium could be measured with radioactive tryptophan, since in these experiments it was not necessary to include tryptophan in the shift-up medium. The increase in efficiency from the normal range to very fast growth conditions could not be measured in the same way, because meaningful tracer experiments cannot be carried out in the very rich medium. However, it could be measured with the same turbidimetric computer-monitored system (Wang and Koch, 1978) described above. In this system the growth rate can be measured so precisely that even over very short periods of time it was possible to measure an increase in the growth rate. It was found that the growth rate increased in less time than that needed for the transit time for ribosomal RNA synthesis (Koch, 1980).

These considerations of changes in efficiency led to the conclusion that the cell adjusts the fraction of working ribosomes so that the rate of protein synthesis per total ribosome does increase progressively with growth rate. Consequently, to test whether there is any other biochemical process rate limiting for growth under the maximal growth rate conditions, the susceptibility of growth to CAP of cells growing in very rich medium was followed turbidimetrically. In studying such rapid growth conditions, we favor the demonstration of an energy limitation. Under these conditions the rate of consumption of energy is high and the surface-to-volume ratio is low because the cells are huge and are growing rapidly. Additionally, the cells may also be approaching their capacity for DNA synthesis and cell division. Still, it can be seen (Fig. 9) with the limited

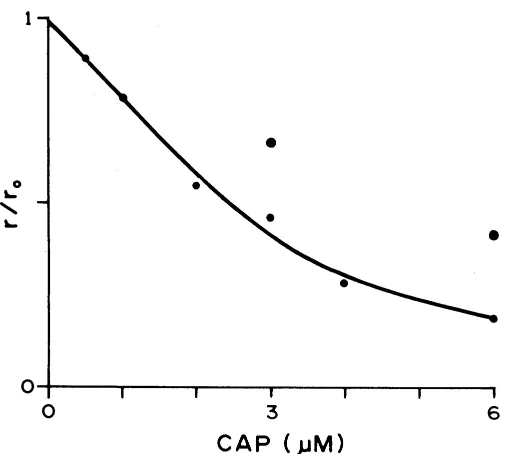

Fig. 9. Dependence of the growth rate on chloramphenicol. The small circles show growth data taken from Harvey and Koch (1980) of growth of *Escherichia coli* ML30 at 30°C in minimal glucose medium. Note that there is no threshold in the action of CAP. The large closed circles are similar data measured during growth in very rich medium as described in the text. Although the data are very limited, these data points also seem to extrapolate to the growth rate at zero drug concentration and therefore imply no threshold in drug action.

data available that there is no threshold, and probably even at the fast growth rate, energy metabolism has not become limiting, contrary to the suggestion of Andersen and von Meyenburg (1980). Moreover, it follows that chromosomal replication and cell division are not limiting at these high growth rates.

This experiment must, however, carry a *caveat emptor* sign. The controls with inhibitors of other cellular processes in which a threshold should be observed have not yet been performed.

Protein Burden

For every stage of evolution, particularly of prokaryotes, a question of paramount importance is the cost in terms of loss of fitness through manufacturing a protein that is not currently useful. The first experiment to measure what has come to be known as the 'protein burden' was that of Novick and Weiner (1957). Their experiment measured how much slower bacteria grew when they made the products of the *lac* operon under conditions in which these proteins served no useful function for the bacteria. They had found a growth medium with a very special property in respect to the regulatory physiology of the *lac* operon. This medium was non-catabolite repressing and contained the non-utilizable inducer thiomethyl-β-D-galactopyranoside (TMG). This organic chemical resembles lactose enough so that it causes the induction of the *lac* operon, but the sulfur atom prevents cleavage by β-galactosidase. A sufficiently low concentration was used such that uninduced cells never became induced. On the other hand, this concentration was sufficiently high so that cells that previously had been induced, and which therefore had galactoside permease, could accumulate the TMG and thereby achieve a much higher internal concentration of the inducer; it was large enough to keep the *lac* operon of induced cells in the derepressed state. Under these conditions, the permease level was maintained high in both daughter cells if their mother had been induced and low if the mother had not. They designated their medium 'maintenance medium.'

When they mixed an induced and an uninduced culture to start a chemostat run with the maintenance concentration of TMG in the medium, the uninduced bacteria gradually overgrew the induced ones. They found that the ratio of types changed by 5% per doubling time, which they attributed to the burden of cellular proteins which were being made but serving no useful function under their conditions (A. Novick, personal communication); in the chemostat the cellular resources that were being funnelled into the 2–3% of cellular protein products would not be available for the synthesis of useful protein, and therefore the growth rate of these cells would be slowed. Their experiment seemingly showed that there was a burden for making protein that the cell could not use. Other experiments addressing the same question by using competition of genetically distinct strains have been carried out by a number of workers since this time. This work has been reviewed recently by Dykhuizen and Hartl (1983) and by Koch (1983b). Therefore, I will not review these papers and instead present a precis of my recent experiments (Koch, 1983b). Protein burden is obviously more critical to the evolution of bacteria than to that of eukaryotic organisms where other cellular and organismal functions would be expected to be more determinative to overall fitness than would simply the economics of protein utilization.

The paradigm for this research was to start from a culture grown under conditions similar to the test conditions, that is, to streak and clone the prototrophic colony on a plate of a composition similar to the test condition to be used in the subsequent experiment. In as few generations as possible, this entire clone was transferred to the corresponding liquid medium and growth was allowed to take place for the fewest number of cell generations to yield just enough cells to start the chemostat culture. This procedure minimized the opportunity for genetic drift. In this way the cells had as little genetic diversity as possible in all regards. The extent of growth from a single cell was such that a few mutations to constitutivity had occurred. These were encouraged to grow faster in the chemostat by a positive selection pressure resulting from the addition of a galactoside that could be attacked by the *lac* operon products but could not serve as an inducer. The substance used was phenyl-β-D-galactopyranoside (PG). The descendents of a cell that had mutated to a constitutive production of *lac* operon products would grow faster as long as PG was present. By manipulating the level and the time, we hoped to enrich the population in constitutive mutants, which subsequently would continue to produce *lac* operon products under conditions in which these products would be unemployed and of no positive use to the cell. Although I had expected to observe a result similar to that of Novick and Weiner, the results turned out to be far different. In a number of preliminary experiments in which a single amendation of the consumable galactoside was made directly to the chemostat, the level of constitutive mutants rose by a factor of 10^4 to 10^5, but after the PG was consumed and/or washed out, the incidence of mutant organisms fell precipitously. The selection disadvantage, relative to the wild type, was observed to be 35–45% per nominal doubling time.

Such values were at least 10-fold higher than expected by the protein burden hypothesis. A logical explanation of these results was that consti-

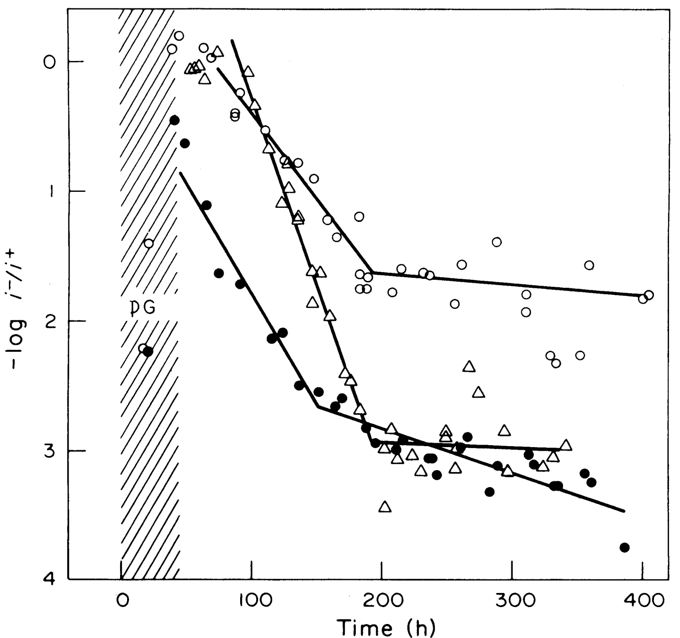

Fig. 10. Selection against *lac* constitutive mutants in chemostats with nominal doubling times of 2 h. A chemostat culture prepared as indicated in the text was challenged with PG to select for constitutive mutants (i^-). After 48 h the PG was removed from the reservoir and the loss of constitutive mutants followed. Each symbol corresponds to an independent experiment.

tutive mutant cells had grown much faster than the wild type when the PG was present, and consequently they had been 'shifted-up' by the presence of an excess carbon source. They were then 'shifted-down' when the galactoside was washed out and/or utilized. Consequently, they would have been at a selection disadvantage simply because of their physiological state.

All subsequent experiments were conducted such that the up-and-down shifts would be minimal. A number of experiments were done with a rapid doubling time chemostat (nominal doubling time, 2 h) and a 24-h selection period at a lower level of the galactoside. In this case also (Fig. 10), the mutant population fell, but at a much slower rate of 5–10% per doubling time. This may have been similar to the results obtained by Novick and Weiner (1957). A second phase was observed in which there was a slow rate of loss; in the three experiments shown, the loss rates were 1.03, 0.10 and 0.32% per doubling time. These surprising results left a doubt as to

the significance of these competition experiments and, in fact, of all those in the literature employing other mutational systems.

A number of alternative explanations were produced and tested in various ways. It could be quickly shown that the *lac*Y gene, or gene product, has an important effect on this system. The *lac*Y codes for galactoside permease and seems to have a deleterious effect on the microorganism in addition to its positive effect. This has been studied by Dykhuizen and Hartl (1978) and earlier workers. Consequently, further studies were carried out with strains not possessing a functional galactoside permease. Initially, studies were plagued by reversion to functional permease formation. In fact, the original isolate of strain ML found by J. Monod was a permease-negative strain which he designated as ML 3. The inducible strain (ML 30, so important in the early phases of molecular biology) was obtained as a revertant from ML 3. This is because the transformation of Y^+ to Y^- and Y^- to Y^+ is normally very facile, and the speed is no doubt the basis of the nearly century-old observation which led to the designation of certain strains of *Escherichia coli* as *Escherichia coli* mutabile in which this change was very frequent. So perhaps these results were not surprising. Therefore, it was necessary to isolate a number of Y^- mutants of ML 30 and select from among them the one with lowest reversion rate. The chosen organism was designated ML 3X. When chemostat experiments were carried out on this strain, it was found that the culture remained essentially permease free and did not revert to the wild-type status. During the challenge period, the ratio of constitutive cells rose progressively from $10^{-7.17}$ to $10^{-4.41}$. Figure 11 shows only the time after the reservoir bottle was changed back to the galactoside-free medium. Also shown is a linear regression line of the data; the slope of this line was $0.15 \pm 0.22\%$, not appreciably different from 0. On the other hand, the upper statistical limit of this slope is certainly less than the fraction of the total protein synthesis that *lac* operon products make—approximately 2 to 3% of the cellular protein. But in any case, the experiment showed that the higher values obtained in the first phase shown in Fig. 10 were attributable to permease involvement. This is probably the reason that Novick and Weiner (1957) had found the value of 5% change per doubling time in their permease-containing cell.

This surprising result, opposite to the very first observations, caused a re-examination of the economic consequences to the cell of making an unneeded protein. There can be no doubt (Koch, 1971; Stouthamer, 1979) that protein synthesis is the major consumer of energy in the cell. Therefore, the manufacture of unneeded protein represents a considerable burden to the cell in terms of energy requirements. Protein also represents a drain on the utilization of carbon and other elements for cell biosynthesis.

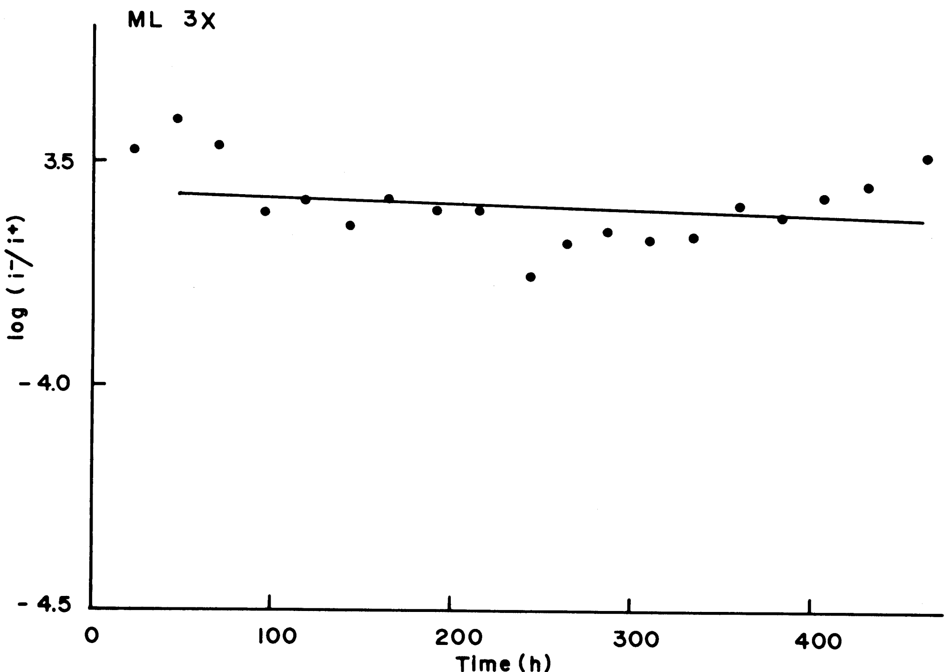

Fig. 11. Competition between *lac* constitutive (i^-) and inducible (i^+) cells. Experiment was similar to that of Fig. 10 except that a stable permease negative strain, ML3X, was used.

For both of these reasons, it should be expected that the burden would have been ameliorated through evolutionary processes to the extent that it could be. Certainly for prokaryotic organisms, regulatory mechanisms for protein synthesis are so sophisticated and precise that it is obligatory to assume that the regulation had evolved to curb even a slight excess protein synthesis which would be deleterious to a cell over many generations, even if the growth slowing had been a small fraction of a percent.

So let us examine in more detail the expected protein burden costs. The calculation to be presented here was previously given in more detail (Koch, 1983b). It is largely based on the tabulations of Stouthamer (1979). Reasonable estimates of the energy needed to form protein and other cellular constituents show the amount of ATP consumed in protein synthesis as 63% of the total energy budget of the cell. For cells grown under similar conditions, the amount of carbon used to form protein amounts to 52.4% of the carbon used to form all cellular constituents. But both energy costs for construction and maintenance must be combined with the capital equipment costs of the manufacture of cell structures. This can be

done if the number of high-energy bonds made from a mole of glucose is known. Unfortunately, this is still debatable; but fortunately, the exact value hardly matters in the final result. If the numerical value of the high-energy bonds found per mole of glucose is designated by p, then $218.8 \times 10^{-4}/p + 38.57 \times 10^{-4}$ mol of glucose are needed for the total cost of formation of protein, and $374.1 \times 10^{-4}/p + 65.8 \times 10^{-4}$ mol of glucose are utilized for all costs for the formation of the total cellular materials in a g dry weight of cells. If the value of p is 36, as in the energy-producing system of mitochondria, the ratio of the two expressions would be 59%. But the bulk of the literature suggests that bacteria are never as efficient as the mitochondrion. If, for example, p is 12 (a probable lower limit for procaryotes), 60% of the cellular resources would be used for protein synthesis. Consequently, independent of energy yield, for every 1% of extra protein, the cell ought to grow 0.6% slower. A similar value would apply for the chemostat cells which had been grown on succinate, although the complete set of numerical estimates is not currently available.

This estimate must be modified to correct for macromolecular turnover. In the computation given above, the costs for mRNA turnover have already been taken into account and are indeed important items. However, there are several components of protein turnover. For *Escherichia coli* grown in minimal medium, approximately 15% of the peptide bonds synthesized under the above conditions are degraded: one-third in the first minute, one-third within a half-life of about 1 h and one-third over a much longer time span (Nath and Koch, 1970). At very slow growth rates, the middle fraction can increase to 20%, but the bulk of the protein is not involved in protein turnover (Koch and Levy, 1955). There are turnover costs for other cell components: DNA repair, loss of outer membrane by blebbing and vesiculation and the turnover of the stable RNA, particularly at slow growth rates (Norris and Koch, 1972). But the quantitative contribution of all of these processes is small, and the ratio of energy plus carbon costs would be adjusted only slightly if accurate values were known, because the correction of the numerator and denominator would each be increased almost proportionately. Therefore, the cost accounting in this regard has probably been accurate, and the burden, based on metabolic pathways, is a 0.6% decrease in growth rate per 1% of protein burden.

It may be necessary to apply a further correction, however, because there is a large discrepancy between experimental and theoretical values of maximum yield of ATP for aerobic growth of *Escherichia coli* on glucose. Stouthamer (1979) quotes experimental values of 6.3 and 8.5 g dry wt cells per mole of high-energy bond obtained in two different ways with *Escherichia coli*, which can be compared with his theoretical value of

28.8. If this large discrepancy were due to an extra energy cost in the form of a futile transport of protons or other ions that are actively moved across the cell membrane but may return by a diffusive or energy-dissipative step, then a smaller value of p is appropriate and, as noted above, would not change the effect of protein burden very much. Although Westerhoff *et al.* (1983) have shown that a low coupling efficiency would allow the cell to grow rapidly, this type of energy loss is not of use to the cell. Such an uncoupled process would simply waste energy without any kinetic advantage. On the other hand, if the discrepancy in growth yields were due to some futile cycle or some as yet unconsidered process subsequent to energy generation and involved some cellular process other than protein formation, then the estimate for total cost for cell dry weight formation should be increased about 3-fold, while the total costs for protein formation was correctly calculated above. This means that if p is as small as 12, the expected protein burden should be about 0.6 times smaller or about 0.4% per 1% extra protein. For gratuitous expression of the *lac* operon containing 2 to 3% of cell protein, a growth rate lowering in the range of 0.8 to 1.2% should have occurred. This is much larger than the statistical limit of the experimental studies reported above. Consequently, it is clear that the expected value lies somewhere between a 0.4 and 0.6% slowing in growth rate for every 1% of gratuitous protein made, and that the experimental effect is smaller than that and currently unexplainable. In spite of its low value, protein burden must be highly significant for prokaryotic evolution and growth. Any mechanism that lowers it below the theoretical limit must be very special and therefore may have evolved more recently than most metabolic processes.

A Problem in Philosophical Biotechnology

Consider a single product produced in large amounts in the world for human consumption: monosodium glutamate (MSG). The commercial fermentation is carried out with *Corynebacterium glutamicum* or *Brevibacterium flavum*. Many other organisms could serve almost as well; Hirose and Shibai (1980) reported 19 wild-type species in various genera that produce large amounts. Moreover, Prescott and Dunn (1954) reported 24 wild-type organisms which carry out almost the entirety of this fermentation, resulting in the production of the closely related α-ketoglutaric acid. Although MSG production is carried out in massive industrial quantities (300,000 tons per year), it was preceded by the cottage industry manufacture of soy sauce and other fermented foods with large MSG contents. Therefore, it is not hard to get organisms to make MSG, and a

strict aseptic technique is not required unless the very highest yield is needed. Production occurs in such large yield that it is clear that this is the major energy-generating process under these conditions. Therefore, the problem of philosophical interest is 'Why in the presence of oxygen would an organism change a substrate such as glucose only into glutamic acid, instead of combusting the glucose all the way to carbon dioxide and water and so obtain a much larger yield of energy for cell growth?' The free energy of combustion of L-glutamate is -2342.5 kJ mol^{-1}, only a little less than the -2872.2 kJ mol^{-1} for the original glucose. So the fermentation squanders resources.

The reason why this is a problem of choice on the part of the organism is that many of the organisms that do so are perfectly capable of carrying the oxidation further. For example, *Escherichia coli* is capable of producing a 40–60% yield of α-ketoglutarate (see Atkinson and Mavituna, 1983) from the glucose supplied, even though the organism has a citric acid cycle and could therefore metabolize glucose all the way to carbon dioxide and water if it so chose. Moreover, Tempest *et al.* (1966) found that in slowly growing ammonia-limited chemostat cultures in the presence of an excess of glycerol, a mutant secreting α-ketoglutarate actually was positively selected. Therefore, we can reject the hypothesis that a high five-carbon acid production is simply due to selection by inscrutable oriental peasants of defective or rare genetic strains that cannot further respire the glutamic acid. So either it is a matter of existential free will on the bacterium's part, or there is some practical biology yet to be appreciated.

The latter hypothesis will be explored here, and six potential reasons as to why an organism would elect to stop short of complete combustion will be discussed. There are two possible variant pathways from hexose to MSG. In one (which I will designate Pathway A), acetyl-CoA is made from one triose derived from glucose while CO_2 is added to the other triose, at either the PEP level or the pyruvate level, to make a four-carbon dicarboxylic acid. Then the two- and four-carbon units are joined to make citric acid. In Pathway B, 1.5 mol of glucose yields 3 mol of acetyl-CoA which, in 2.5 turns of the glyoxylate shunt, creates a net molecule of citrate. In either case the citrate is converted to isocitrate, which is then decarboxylated to α-ketoglutarate and aminated to form glutamate.

Cost of Enzyme Production

One hypothesis to account for MSG production is that the further metabolism would require an enzyme expensive to the cell's economy. It is indeed true that α-ketoglutaric dehydrogenase is a very complex multisubunit enzyme that requires three vitamins (thiamine, riboflavin and

lipoic acid) in its structure and involves a good deal of genetic information and expenditure of energy and materials for the biosyntheses to create the complex holoenzyme. Thus, organisms completing the oxidation need to be capable of doing the difficult and expensive work of synthesizing this complex enzyme. However, this does not appear to be a highly cogent reason, because in any case the cells must produce a very similar enzyme with some of the same parts which are needed in the production of acetyl-CoA. But in the support of this hypothesis is the finding of Shiio *et al.* (1961) that the enzyme is absent from the high glutamic acid producer *Brevibacterium flavum*. Consequently, a possible but not universal answer is that organisms that fail to be able to finish the Krebs cycle do so because the capital equipment is too expensive. Then they must dispose of something; frequently in a high-nitrogen environment, it is glutamic acid.

Effective Use of Oxygen

A second possible reason is that five-carbon acid production is the optimum strategy when the oxygen tension is low. This is suggested by the fact that high glutamic acid yields require micro-aerophilic conditions. In laboratory experiments (Hirose and Okada, 1979) the yield of glutamate became zero both when no oxygen was present or when a very high partial pressure of oxygen was maintained during growth. In the latter case there was a high yield of undesirable CO_2. We should note that while the production of glutamic acid is called a 'fermentation' by biotechnologists, it is certainly not fermentation as classified by microbiologists, since it is not anaerobic. It does require oxygen, and it must yield energy to the organism by way of oxidative phosphorylation. So it might be argued that this pathway could be an intermediate between a completely anaerobic fermentation, such as homolactic fermentation, and fully aerobic citric acid cycle—intermediate in the sense that an intermediate fraction of energy is derived from substrate-level phosphorylations. In the first line of Table 2 the percentage of the energy derived from substrate phosphorylation of the total energy available from metabolism is given. It can be seen that this percentage is virtually the same for either of the two variant modes of glutamic acid production as it is for the total combustion via the citric acid cycle. So this explanation appears to be unsupported by the known energy-coupling processes.

A slight variant of this hypothesis is that fewer electrons, which must be oxidized by respiration, are formed per substrate level phosphorylation event when carrying out an incomplete fermentation. But a simple counting up of the electrons per energy unit made available to the cell in the

Table 2. *Comparison of metabolic pathways from glucose to lactate, to glutamate or to CO_2*

Per mole glucose	Lactate[a] via Embdem–Meyerhoff–Parnas	Glutamate A[b] via CO_2 fixation	Glutamate B[c] via glyoxylate shunt	CO_2[d] via Krebs citric acid cycle
Percent energy derived from substrate level phosphorylations	100	10	10	11
Number of substrate level phosphorylations	2	1	1.5	4
Free energy dissipation[e] $\Delta G'_{ox}$ (pH 7)				
In carbon transformation (kJ)	−209.80	−228.8	−233.82	−243.82
In further oxidation (kJ)	−47.8	−2.7	−2.7	0
Electrons produced (at levels of NADH + H⁺)	0	6	12	24

[a] $C_6H_{12}O_6 \rightarrow 2C_3H_5O_3^- + 2H^+ + OH°$.
[b] $C_6H_{12}O_6 + NH_4^+ \rightarrow CO_2 + C_5H_8O_4N^- + 2H^+ + 6H°$.
[c] $1.5C_6H_{12}O_6 + NH_4^+ + 3H_2O \rightarrow C_5H_8O_4N^- + 4CO_2 + 2H^+ + 18H°$.
[d] $C_6H_{12}O_6 + NH_4^- \rightarrow 6CO_2 + 24H°$.
[e] Free energy dissipation expressed as $\Delta G'$ (pH 7) in kJ per mole of two-carbon compound transformed. This is the energy available in oxidizing the compound with NAD⁺. Each pair of electrons at this level can result in 219 kJ of energy dissipated in the electron transport chain with concomitant energy production to an extent depending on the coupling to phosphorylation. A mole of high-energy bonds $\Delta G'$ (pH 7) is worth 34.5 kJ. To convert values given in kJ to kcal, divide by 4.185.

form of high-energy phosphate bonds (see Table 2, line 2) indicates that the yield in these terms is also the same for the Krebs cycle complete oxidation as for glutamic acid production by either pathway. So an explanation that draws an analogy to the production of smoke from an overchoked engine does not seem to fit the facts.

Energy Realization during Disposal of By-product

A third possibility is that the production of glutamate *per se* may be favorable to the cell's energy economy simply in the process of extruding the glutamic acid from the cell. It is already known that the anaerobic lactic acid bacterium *Streptococcus cremoris* makes additional energy available for its biosynthetic processes as the result of forming and excreting lactic acid (Michels *et al.*, 1979; Konings *et al.*, 1981). The lactic acid leaves the cell flowing down its thermodynamic gradient. The cell's mechanism for the transport across the cell membrane (a symport carrying undissociated lactic acid plus a proton) forces the extrusion of an additional

proton into the environment. This extruded proton both creates a charge difference across the membrane and increases the acidity of the environment. Both increase the protonmotive force. Consequently, the cell acquires an additional gratuitous metabolic energy source when those protons are allowed to re-enter the cell, causing the production of ATP while so doing. Therefore, if the cell possesses the appropriate transport mechanisms that functions as a symporter for protons and metabolic products, it is possible that either glutamic acid formation or α-ketoglutarate formation could be a windfall for energy production without additional O_2 consumption. If the cells get an additional proton extruded with the fermentation product as it is dumped from the cell, the result is an additional production of a high-energy phosphate bond that would make α-ketoglutarate and/or glutamic acid formation and excretion favorable under micro-aerophilic conditions.

Use of By-product for Osmotic Stabilization

It is a well-known observation in the technical literature that a sub-maximal level of biotin is needed for maximal glutamic acid production (Tanaka *et al.*, 1960) and that increased yields can be obtained by adding fatty acids or detergents to media based on beet juice, which contains an abundance of biotin. Moreover, it has been found that by treatment of growing cultures with a low level of penicillin, glutamic acid productivity can be increased (Somersham and Philips, 1961). It has been argued from these facts that the production of glutamic acid is dependent on a partially defective membrane, so that the cell leaks glutamic acid instead of extending the metabolism to form carbon dioxide and water. This argument, while repeated often, may not be the entire story. In the fermentation media usually used, potassium is relatively scarce (<10 m*M*) and the solute concentration relatively high. There is a possibility that organisms in these types of growth media have to solve, among others, an osmotic problem, and to do so they may employ a trick well studied in other instances. When the osmotic environment is high, many organisms will accumulate high levels of potassium to raise the internal osmotic pressure so that it is higher than the external one. For example, *Escherichia coli* has three pumping systems for potassium that can be called into play to accumulate potassium (Laimins and Epstein, 1980). One of these is clearly responsive to the cell's osmotic pressure relationships. But organisms have alternatives to accumulating potassium. A frequent one is to manufacture or accumulate high levels of proline or glutamic acid (Tempest *et al.*, 1970; Csonka, 1981). So it might be speculated that through normal regulatory channels those organisms used industrially are attempting to increase the internal osmotic pressure by virtue of synthesizing glutamic acid or α-ketoglutarate. Under such conditions, a small degree of leakage of these substances through the membrane is due to a weakening of the peptidoglycan sacculus as a result of either a biotin deficiency or the effects of external fatty acids. Another possibility is that insufficient peptidoglycan allows the cell to swell and leak additional components. Note that the suggestion of leakage is partially antagonistic to the energy-coupling argument made in the previous section.

Lack of Profit Incentive at Critical Stages

Consider the thermodynamics of the relevant biochemical pathways. In an important sense, one should separate conceptually the energetics of the detailed transformations of carbon skeletons to form the reducing equivalents in the form of either NADH + H^+, NADPH + H^+ or succinate from the subsequent oxidation of these species. This separation is necessary because the energetics of the oxidative phosphorylation are not coupled to the chemical re-arrangements; the rate of biochemical processing of the carbon skeletons is a function of the free energy changes involved in the carbon skeleton transformations to the level where the hydrogens are made available to the electron transport carrier system and is independent of the value of subsequent energy transformations.

It is important to point out that the redox chain does not drive the carbon skeleton transformation in respiratory metabolic pathways the way the early phosphorylations do in the Embden–Meyerhof–Parnas fermentation. An exergonic process cannot effectively 'pull' a biochemical process, but it can, from a kinetic point of view, effectively drive one. This means that if steps in the overall carbon chemistry proceed with a lower expenditure of free energy, the entire process will occur at a slower rate.

In Table 2 these free-energy values have been calculated from the data tabulated by Metzler (1977). It can be seen that the free energy forcing the carbon skeleton transformations is virtually the same in the full Krebs cycle as it is in the processes leading to glutamic acid. This means that there is less free energy per step to be dissipated in the carbon compound transformation for the full Krebs cycle, which involves several additional enzymatic steps with unfavorable free energy. Consequently, less energy is available to drive the production of the hydrogen equivalents. The same conclusion can be made more directly by noting that there is almost no energy available from the complete oxidation of the glutamate under physiological conditions, which would be disrupted in the carbon skeleton transformation. This explanation may account, in the final analysis, for

the choice of pathways these microorganisms take; that is, the pathway in which the carbon skeleton transformation has a larger negative free energy on average per step, even though it results in less trapped energy, may frequently be the appropriate choice for an expedient microorganism (Westerhof *et al.*, 1983).

It Is More Expedient to Exploit Than to Conserve

The final possibility in our metaphysical discussion of the economics of energy production is that the bacterium finds it more expedient to exploit than to conserve. It could be argued that the microorganisms have acquired enough energy by the production of glutamate and that failure of further oxidation might be explained by the well-known phenomena of overflow metabolism (Neijssel and Tempest, 1975, 1976, 1979; Tempest and Neijssel, 1976). They found that under certain chemostat culture conditions it is easy to demonstrate that a microorganism might very efficiently metabolize and use the energy from glucose oxidation when under glucose-limiting conditions, but the same organism will be a spendthrift and use up and despoil glucose well beyond its energy needs when limited in its growth by some other nutrient. Possibly, the cell does not have adequate controls to shut down its uptake and utilization of the external substrate to the austere, puritanically efficient level; its mouth is bigger than its stomach. Therefore, the cell either must produce energy that must be wastefully dissipated in the form of futile cycles of consumption of un-needed ATP or it must not generate the metabolic energy, obliging it to secrete partially oxidized compounds in the medium. This hypothesis suggests that microorganisms (like people) have simply not been selected in the past for efficient regulation of utilization of available resources. Thus the large glutamic acid production may simply be a striking example of such prodigal and human-like behavior.

A quite extreme example of a microorganism which carries out only a portion of a pathway concerns the metabolism of adenosine by *Escherichia coli* growing in a glucose minimal salts medium (Mans and Koch, 1960). These cells are potentially capable of utilizing the nucleoside for its amino group, it sugar residue, or the purine base. Measurement of transformation of the substrate by growing cultures showed that the organisms do take up adenosine and excrete inosine at a rate 45 times greater than their need for purines. Secondarily, they take up inosine, remove the ribose and excrete hypoxanthine 10 times faster than their need for purines. Hypoxanthine, like inosine, or adenosine would be taken up for the cellular adenine, and most of the guanine, requirements at exactly the rate needed to supply purines by the salvage pathway, and the *de novo* pathway would be almost fully repressed (Koch, 1955).

Another Test Case

At the present time I believe that there is no way to critically choose between the six classes of possibilities that have been enumerated for MSG production; they need to be tested. However, an educated guess supports the thermodynamic reasoning. I justify this choice on the basis of another example: the ecology and thermodynamics of acetic acid production. Under anaerobic conditions, many organisms can create a supply of ethyl alcohol from fermentable sugars. Alcohol formation is one of the almost equally useful metabolic options in terms of energy generation. Subsequently, under an oxygen-containing environment, acetic acid bacteria convert that ethanol to acetic acid, biomass and sometimes great quantities of polysaccharide. But seemingly, these organisms, too, could have oxidized the acetic acid all the way to carbon dioxide and water. It is not justifiable to claim that today's acetic acid bacteria do not have the enzymes to carry out the Krebs cycle, because over evolutionary time organisms would have arisen that made much more use of this available energy reserve than the acetic acid bacteria do. So why has this not happened? The answer is very easy to formulate on thermodynamic grounds (see Table 3). The point is simply that there is not enough free energy dissipation from acetic acid to CO_2; in fact, the reaction is unfavorable to the cells. Plenty of ATP can be produced from the eight electrons that could be realized in the further oxidation, but given the textbook biochemical pathway there is no mechanism to plow that needed ATP

Table 3. *Energetics of ethanol oxidation*

	Ethanol[a] to acetic acid	Ethanol[b] to acetate	Ethanol[c] to CO_2
Free energy dissipation[d]	−13.9 kJ	−26.7 kJ	−4.6 kJ
In transformation			
Number of electrons	4	4	12
In further oxidation	+9.3 kJ	+22.1 kJ	0 kJ
Number of electrons	8	8	0

[a] $C_2H_5OH + H_2O \rightarrow C_2H_4O_2 + 4H^\circ$.
[b] $C_2H_5OH + H_2O \rightarrow C_2H_3O_2^- + H^+ + 4H^\circ$.
[c] $C_2H_5OH + 3H_2O \rightarrow 2CO_2 + 12H^\circ$.
[d] Free energy dissipation expressed as $\Delta G'$ (pH 7) in kJ per mole of two-carbon compound transformed. This is the energy available in oxidizing the compound with NAD^+. Each pair of electrons at this level can result in 219 kJ of energy dissipated in the electron transport chain with concomitant energy production to an extent depending on the coupling to phosphorylation. A mole of high-energy bonds $\Delta G'$ (pH 7) is worth 34.5 kJ. To convert values given in kJ to kcal, divide by 4.185.

energy back into driving the pathway to complete the transformation of the carbon skeletons. Therefore, it is not likely that organisms would have arisen to oxidize ethanol to free acetic acid in an acidic environment as their sole or major source of energy. While the thermodynamics are clear that much oxidative metabolism of ethanol stops at acetic acid, there are some oxidations that go on to CO_2; they must have a different energy-coupling arrangement. If an acidic, aerobic habitat with acetic acid as the major carbon source had been abundant in the past, a different metabolic scheme might have arisen—one that apportioned a larger fraction of the available free energy of oxidation to the carbon transformation step and less to the electron transport aspect. This might have favored more complete combustion of a single substrate within a single organism, given ethanol and O_2, to create more cells and less by-product.

Conclusion

I have tried to establish that microbial ecology and evolution is a subdiscipline of economics. Microorganisms engage in capitalistic free enterprise where the profit motive is not the important thing—it is the only thing. Altruism is rarely apparent, and examination of candidate cases shows that self-interest of the parties is served. A good example is *Methanobacillus omelianskii* which is now known to be a consortium of two bacteria. One makes a profit only if its waste product (H_2) is efficiently removed from the environment; the other can show a profit margin only when H_2 is available without cost.

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2

Starvation, Survival and Energy Reserves

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Introduction

In their natural environments microorganisms are subjected to fluxes of nutrients which may encompass conditions of surfeit, sub-optimal amounts or the complete absence of substrates. It is vital, therefore, that they should be able to survive the periods of deprivation and recover rapidly when the supply of nutrients improves. The present contribution addresses itself to the factors influencing the survival of bacteria under conditions of nutrient starvation and in the absence of other overt stresses.

Factors involved in the survival of microorganisms under starvation conditions are complex and frequently interactive, although in the final analysis it is likely to be the exhaustion of an intracellular source of energy which results in death of the organism. Parameters that have been implicated in the survival of vegetative bacteria include the substrates available for endogenous metabolism, the possession of storage compounds, the energy of maintenance requirement, the adenylate energy charge and the preservation of a membrane potential. Some bacteria are able to invoke the specialised mechanisms of spore or cyst formation to enable them to withstand adverse environmental conditions and there is some evidence to suggest that oligotrophic bacteria may assume dormant forms to aid their survival, for example, in the marine environment; the former mechanisms are excluded from the present survey, which is confined to vegetative forms, and the latter is an aspect dealt with in Chapter 4 of this volume (Morita).

The majority of bacteria are able to survive, often for prolonged periods, in the absence of nutrients—conditions of so-called 'starvation sur-

BACTERIA IN THEIR NATURAL ENVIRONMENTS

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