The Effects of Cooperativity and Growth Yield Variation on the Kinetics of Nitrogen or Phosphate Limited Growth of *Chlorella* in a Chemostat Culture

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Accounting for a cooperativity effect in substrate uptake by a microbial culture leads to a Hill type of equation for the specific rate of substrate uptake (Q), that is,

$$Q = Q_m S^n / (S^n + K_{sh})$$

where S is substrate concentration, n is the Hill number and K_{sh} is a constant. For substrates such as N or P, which are conserved in the biomass, the substrate content of the biomass (α) will vary according to the relation

$$(\alpha - \alpha_0) / (\alpha_m - \alpha_0) = S^n / (S^n + K_{sh})$$

where α_0 is the minimum substrate content of the biomass, occurring when $S \rightarrow 0$, and α_m is the maximum substrate content of the biomass, occurring when the biomass is saturated with substrate. The specific growth rate is given by

$$\mu = \mu_m S^n / (S^n + LK_{sh})$$

where L is a constant given by α_0/α_m . If the substrate is conserved, $\alpha_0 = 1/Y_0$ and $\alpha_m = 1/Y_m$ where Y_0 and Y_m are the maximum and minimum yields from the substrate, respectively.

Experimental tests of these relations applied to N (urea)-limited growth of *Chlorella vulgaris* in chemostat cultures showed satisfactory agreement between the results and the theory. For N uptake the growth constants were $n = 5.5$, $L = 0.47$, $K_s = (LK_{sh})^{1/n} = 82 \mu\text{g N l}^{-1}$, $K'_s = K_{sh}^{1/n} = 94 \mu\text{g N l}^{-1}$. The apparent departure of L from unity in N-limited growth can be accounted for by starch storage in the biomass.

For P (phosphate)-limited growth, $n = 1.3$, $L = 0.15$ and K_s was about $28 \mu\text{g P l}^{-1}$. A discrepancy was found between the K_{sh} value for P uptake obtained from the Q data and that from the α data. This discrepancy may be attributed to phosphate storage in the biomass, which is not allowed for in the model.

INTRODUCTION

The principal mathematical expression used to relate specific microbial growth rate (μ) to the concentration of growth-limiting substrate (S) is the equation of Monod (1942)

$$\mu = \mu_m S / (S + K_s) \quad (1)$$

where μ_m is the maximum value to which μ tends as S increases. The Monod equation was empirically based on the assumption that the growth yield (Y) is a constant. If the

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Fig. 1. Graphs of relations $\mu = \mu_m S / (S + K_s)$ (---) and $\mu = \mu_m S^5 / (S^5 + K_{sb})$ (—), where $\mu_m = 1$, $K_s = 1$ and $K_{sb} = 1$. The values of S and μ are in arbitrary units.

equation is derived by application of the Michaelis–Menten theory of enzyme kinetics, we first obtain (Pirt, 1975, p. 8)

$$Q = Q_m S / (S + K_s) \quad (2)$$

where Q is the specific rate of uptake of growth-limiting substrate. Since $Q = \mu / Y$ and $Q_m = \mu_m / Y$, if Y is constant, we derive equation (1).

In general, it is technically difficult to test any equation relating specific growth rate and growth-limiting substrate concentration because the limiting concentrations are so small. However, it is clear that the Monod equation has its limitations and various attempts have been made either to refine the equation or to develop an alternative. Shehata & Marr (1971) found deviations which they accounted for by assuming the existence of more than one substrate uptake mechanism with different substrate affinities for each mechanism. A new equation was developed by Dabes *et al.* (1973) from a model of a more complex enzyme system proposed to represent biomass growth. This equation included a third constant, besides μ_m and K_s , which essentially represents $d\mu/dS$ at low substrate concentrations.

To obtain greater flexibility in the Monod equation, Moser (1958) replaced the first power of S in equation (1) by an arbitrary power, r :

$$\mu = \mu_m S^r / (S^r + K^r) \quad (3)$$

where K is a constant. One effect of this modification with $r > 1$, for which there is justification (Powell, 1967), is to make μ approach its asymptotic value, μ_m , more rapidly than it does in the Monod equation (see Fig. 1). Also the graph of equation (3) with $r > 1$ is convex towards the S -axis near the origin, that is, it resembles a sigmoid rather than a hyperbolic curve. The main objection to equation (3) was that the introduction of the variable power, r , was purely arbitrary and not justified by a physiological explanation. However, recent progress in enzymology makes it clear that anomalous reaction order with respect to substrate (when the plot of enzymic reaction velocity versus substrate concentration gives a sigmoid curve) is not exceptional and indicates that there is interaction between binding sites on the enzyme molecule (Gutfreund, 1972). Such ‘cooperative effects’ resulting from interaction between identical ligands have been termed ‘homotropic’ (Gutfreund, 1972). By a homotropic effect the substrate affinity of the enzyme is progressively increased after binding each substrate molecule. The embodiment of the cooperativity effect in enzyme reactions (Kargi, 1977) leads to an equation identical to that proposed by Moser (1958). However, no experimental evidence for cooperativity effects in substrate uptake by growing microbes has been reported previously.

Another defect of the Monod equation is that it does not allow for variation in the growth yield (Y). In the case of growth limited by the energy source, the existence of maintenance energy causes the growth yield from the energy source to decrease with the specific

growth rate. The effect that this growth yield variation will have on the growth rate equation was indicated by Powell (1967).

Both Droop (1974) and Nyholm (1976) drew attention to the variation in growth yield when growth is limited by so called 'conserved substrates' such as sources of P and N, which, in contrast to carbon and energy sources, provide an element that is conserved in the biomass. Nyholm (1976) pointed out that the growth yields from conserved substrates in general increase with decrease in specific growth rate. Consequently Droop (1974) and Nyholm (1976) each proposed a different empirically based relation between specific growth rate and growth yield (Y) or its reciprocal (α), which is the content of conserved substrate in the biomass. Nyholm (1976) went so far as to state that the content of conserved substrate in the biomass (α) controlled the growth rate and that 'no direct relationship exists between the specific growth rate and the extracellular concentration of growth-limiting substrate'.

The experiments of Droop (1974) and Nyholm (1977) were concerned with algal cultures and cell number or total cell weight was used as the criterion of biomass. Recently Watts Pirt & Pirt (1977) have shown that the apparent increase in growth yield with decrease in specific growth rate in N-limited cultures of *Chlorella vulgaris* can be accounted for by starch storage in the cells. With 'real biomass' defined as total biomass *minus* the stored starch, the growth yield from N based on real biomass was constant.

We have now investigated the relation between the specific growth rate of *C. vulgaris* and growth-limiting concentrations of two conserved substrates N (from urea) and P (from phosphate) in steady states of chemostat cultures. The concentration of biomass was kept as low as possible by restricting the substrate concentration (S_f) in the feed medium in order to facilitate rapid removal of the biomass (in < 1 s) by filtration of samples. *Chlorella vulgaris* was used as the test organism principally because of its potential importance for the utilization of solar energy. Because biomass density was so low, it was not feasible to estimate the cell dry weight directly or determine the starch in the cells and so calculate the real biomass. Consequently we have used absorbance or cell number as the measure of biomass. To allow for the effect of growth yield variation on the growth rate equation, we derived a modification based on first principles. This paper presents the new growth rate equation together with experimental evidence for it.

THEORY

According to the Kargi (1977) model, the reaction between biomass and the growth-limiting substrate is represented as

where X_t represents the biomass unit which can react with n molecules of growth-limiting substrate (S) to form one unit of biomass-substrate complex (XS_n). It is assumed that the biomass is always saturated with substrates other than the growth-limiting one. The effects of these other substrates are assumed to be incorporated into the constants of the growth rate equation. The Kargi model leads to the following expression for the specific rate of substrate uptake (Q)

$$Q = \frac{Q_m S^n}{S^n + K_{sh}} \quad (5)$$

where Q_m is the specific rate of substrate uptake when the biomass is saturated with substrate ($X = XS_n$). If the growth yield Y does not vary with μ then we may substitute $Q = \mu/Y$ and $Q_m = \mu_m/Y$ in equation (5), which leads to the equation for μ derived by Kargi. When $n = 1$ the growth rate equation reduces to the Monod equation.

Effect of growth yield variation. The effect of growth yield variation on the relation of growth rate to substrate concentration is derived in the following way. We make the simplifying assumption that the biomass consists of two parts. One part X_0 has the minimum content (α_0) of conserved substrate. The second part X_m has the maximum content (α_m) of conserved substrate. At zero growth rate it is assumed that the biomass is entirely in the X_0 form and at maximum growth rate it is entirely in the X_m form. No cause

of the growth yield variation is implied. Storage of starch in the X_0 form, but not in X_m could account for the effect. It could also be accounted for by an increase in the requirement for the substrate, with increase in specific growth rate, for instance, more P could be required to increase the RNA content of the biomass. By taking the substrate balance we obtain

$$S_r = S + \alpha_0 X_0 + \alpha_m X_m \quad (6)$$

where S_r is the concentration of growth-limiting substrate initially supplied, S is the residual substrate concentration, α_0 and α_m are the substrate contents in the biomass forms X_0 and X_m respectively (X_0 and X_m also represent the weight of each form). The total biomass is given by

$$X = X_0 + X_m \quad (7)$$

Substituting for X_0 in equation (6) and putting $\Delta S = S_r - S$ we obtain

$$X_m = \frac{\Delta S - \alpha_0 X}{\alpha_m - \alpha_0} \quad (8)$$

The fraction of the biomass saturated with the substrate is given by

$$\frac{X_m}{X} = \frac{\alpha - \alpha_0}{\alpha_m - \alpha_0} \quad (9)$$

where $\alpha = \Delta S/X$. We now suppose, as in the Kargi model, that the specific rate of substrate uptake (Q) is proportional to X_m/X , that is, $X_m/X = Q/Q_m$. Then from equations (5) and (9) we obtain

$$\frac{\alpha - \alpha_0}{\alpha_m - \alpha_0} = \frac{S^n}{S^n + K_{sh}} \quad (10)$$

Equation (10) can be tested by putting it in the form

$$\log\left(\frac{\alpha - \alpha_0}{\alpha_m - \alpha_0}\right) = n \log S - \log K_{sh} \quad (11)$$

and making a Hill plot, that is, $\log[(\alpha - \alpha_0)/(\alpha_m - \alpha)]$ against $\log S$.

The biomass growth rate is determined by putting $Q = \mu\alpha$ in equation (5) and substituting for α by means of equation (10). This leads to

$$\mu = \frac{\mu_m S^n}{S^n + L K_{sh}} \quad (12)$$

where $\mu_m = Q_m/\alpha_m$ and $L = \alpha_0/\alpha_m$. The K_s value, that is, the substrate concentration at which $\mu = 0.5 \mu_m$ is given by $(L K_{sh})^{1/n}$. The saturation value for uptake of substrate differs from K_s and is given by $K'_s = K_{sh}^{1/n}$.

METHODS

Symbols and units. These were as follows unless otherwise stated.

- K_s , saturation constant for growth ($\mu\text{g substrate l}^{-1}$).
- K'_s , saturation constant for substrate uptake ($\mu\text{g substrate l}^{-1}$).
- K_{sh} , $(K'_s)^n$.
- L , α_0/α_m (dimensionless).
- $\ln$, natural logarithm.
- n , Hill number (dimensionless).
- Q , specific rate of substrate uptake [$\mu\text{g substrate (10}^6 \text{ cells)}^{-1} \text{ h}^{-1}$]; subscripts N and P specify nitrogen and phosphorus uptake, respectively.
- Q_m , theoretical maximum specific rate of uptake of substrate [$\mu\text{g substrate (10}^6 \text{ cells)}^{-1} \text{ h}^{-1}$].
- S , substrate concentration ($\mu\text{g l}^{-1}$).
- X , total biomass concentration ($10^6 \text{ cells ml}^{-1}$).
- Y , growth yield [$10^6 \text{ cells (}\mu\text{g substrate)}^{-1}$].
- α , content of substrate in the biomass [$\mu\text{g substrate (10}^6 \text{ cells)}^{-1}$]; subscripts N and P specify nitrogen and phosphorus contents, respectively.
- α_0 , minimum content of substrate in the biomass [$\mu\text{g substrate (10}^6 \text{ cells)}^{-1}$].
- α_m , maximum content of substrate in the biomass [$\mu\text{g substrate (10}^6 \text{ cells)}^{-1}$].
- μ , specific growth rate (h^{-1}).
- μ_m , theoretical maximum specific growth rate (h^{-1}).

Organism and growth. The Sorokin strain of *Chlorella vulgaris* 211/8K (Culture Collection of Algae and Protozoa, Cambridge) was grown and maintained at 37 °C on medium A8 solidified with agar with 5% CO₂ in the gas phase and under continuous light. Both stock and chemostat cultures were, from time to time, tested for possible contamination by means of light microscopy and plating on peptone nutrient agar. The basic culture medium was the defined medium A8 of Watts Pirt & Pirt (1977), except that the NaVO₃ concentration was 0.05×10^{-3} g l⁻¹. To achieve phosphate-limited growth, phosphate was decreased to between 0.08 and 4.4 mg P l⁻¹. Urea-limited growth was obtained with urea at between 1.7 and 10.0 mg urea l⁻¹. The medium in the chemostat was equilibrated with 5% CO₂ in the gas phase.

The chemostat culture apparatus with illuminated growth vessel was as described by Watts Pirt & Pirt (1977). The pH value of the cultures, including those with phosphate-limited growth, stayed in the range 6.2 to 6.5 under all conditions without instrumental control.

Analytical procedures. The biomass was estimated either turbidimetrically at 570 nm or by counting cells with a Coulter counter (Coulter Electronics) with a 50 µm orifice probe. The ratio of turbidity to cell count did not vary significantly at all dilution rates tested (0.02 to 0.25 h⁻¹) with either urea- or phosphate-limited growth: an absorbance of 0.1 corresponded to 1.1×10^8 cells ml⁻¹ and proportionality was maintained up to at least 0.3. The cell dry weight also did not vary significantly with the specific growth rate (M. Watts Pirt, unpublished work): 1 mg total dry wt $\approx 1.2 \times 10^8$ cells. Phosphate concentrations in the medium down to 10 µg l⁻¹ were determined colorimetrically by the method of Chen *et al.* (1956). Urea was determined by the urease method with the Boehringer kit. The sensitivity of the method was increased sixfold by doubling the reagent concentration and increasing the ratio of sample volume to reagent volume fivefold. The lowest detectable level of urea nitrogen was 20 µg l⁻¹. The method depends upon ammonia liberated from the urea, hence any free ammonia in the culture medium would also appear as urea. The urea uptake was taken to be ($S_r - S$) where S_r was the concentration in the medium feed and S was the residual concentration in the medium.

Cell-free culture medium for urea or phosphate assays was immediately separated from a culture sample by rapid filtration through a membrane filter (0.45 µm, Oxoid).

RESULTS

Urea-limited growth

Residual urea in chemostat steady states could be determined directly at all tested dilution rates with nitrogen-limitation. The biomass nitrogen was assumed to be the difference between the urea nitrogen supplied and the residual urea nitrogen. Nitrogen contents of the biomass are given in Fig. 2. As the specific growth rate increased, the value of α_N increased towards the maximum value α_m . The maximum nitrogen content of the biomass was obtained when the culture was phosphate-limited. The α_N values with phosphate-limited growth at different specific growth rates had a considerable scatter, for unknown reasons, but remained approximately at the maximum value (Fig. 2).

Both the specific growth rate and specific N uptake rate were sigmoidal functions of the urea nitrogen concentration (Fig. 3*a, b*). The Hill plots (Fig. 4) based either on Q (equation 5) or on α (equation 11), for nitrogen-limited growth, were identical. The growth constants are shown in Table 1. The values of the Hill number (n), K_s and K'_s for nitrogen-limited growth could be obtained equally well from either the Q or the α values. The data points in Fig. 3(*a, b*) agree satisfactorily with the theoretical curves of equations (5) and (12), respectively. The pronounced sigmoid form of the curve gives rise to the large value for n .

Phosphate-limited growth

The residual steady-state phosphate concentration in a chemostat culture was too low to be determined directly and was therefore determined by the method of Button (1969), which makes use of the relation $\tilde{X} = YS_r - Y\tilde{S}$. The concentration of phosphate in the reservoir (S_r) was checked by analysis. Each straight line of $\tilde{X}$ against S_r in Fig. 5 is a least-square fitted plot at a given value of D with different phosphate concentrations (S_r) in the medium feed. Extrapolation of the line to $X = 0$ gives the value of the residual substrate concentration S .

With phosphate-limited growth, the P content of the biomass increased relatively slowly

Fig. 2. Nitrogen content of biomass (α) as a function of specific growth rate (μ) in steady states of chemostat cultures of *Chlorella vulgaris*: ●, with N-limited growth; □, with P-limited growth.

Fig. 3. (a) Specific growth rate (μ) and (b) specific rate of nitrogen uptake (Q) as functions of substrate nitrogen concentration (S) in steady states of N-limited chemostat cultures of *Chlorella vulgaris*. The lines drawn are the theoretical curves of the relations $\mu = \mu_m S^n / (S^n + LK_{sh})$ and $Q = Q_m S^n / (S^n + K_{sh})$, where n , μ_m , Q_m , L and K_{sh} have the values given in Table 1.

with μ until about 80% of μ_m was reached (Fig. 6); therefore α_p must have increased rapidly to attain the maximum value when phosphate was no longer growth-limiting. With nitrogen-limited growth, α_p showed a considerable scatter about the maximum value for unknown reasons (Fig. 6).

Both the Q and α functions of P-limited growth (equations 5 and 10) gave straight line Hill plots (Fig. 4). The slopes of the Hill plots from α_p and Q_p were identical, with a Hill number (n) of 1.3. In contrast to the N-limited cultures, the α and Q plots differed significantly in the intercepts which give K_{sh} . The growth constants for P uptake are given in Table 1.

The plots of μ and Q_p against P concentration in P-limited cultures are shown in Fig. 7(a,b); that of μ against P (Fig. 7a) deviated significantly from the theoretical curve. This deviation and the discrepancy in the K_{sh} values for P uptake obtained from the α and Q curves may be attributed to another effect on phosphate uptake, which has not been allowed for, namely phosphate storage possibly as polyphosphate. This means that instead

Table 1. Growth constants of *Chlorella vulgaris* 211/8K for N (urea) uptake and P (phosphate) uptake

Parameter	For N uptake	For P uptake	
μ_m (h^{-1})	0.25	0.25	
Q_m [μg element (10^6 cells) $^{-1}$ h^{-1}]	0.310	0.0962	
α_0 [μg element (10^6 cells) $^{-1}$]	0.584	0.0588	
α_m [μg element (10^6 cells) $^{-1}$]	1.235	0.385	
$L = \alpha_0/\alpha_m$	0.47	0.152	
	From Q_N or α_N	From Q_P	From α_P
Hill number (n)	5.5	1.32	1.32
$K'_s = K_{sh}^{1/n}$ (μg l^{-1})	94.2	116	200
$K_s = (LK_{sh})^{1/n}$ (μg l^{-1})	82.1	28	52

Fig. 4

Fig. 5

Fig. 4. Hill plots of either $y = \ln [Q/(Q_m - Q)]$ or $y = \ln [(\alpha - \alpha_0)/(\alpha_m - \alpha)]$ versus $\ln S$ for P and N uptake in steady states of chemostat cultures of *Chlorella vulgaris*: $\blacktriangle$, from Q_P data for P-limited growth; $\square$, from α_P data for P-limited growth; $\blacksquare$, from Q_N data for N-limited growth; $\bullet$, from α_N data for N-limited growth.

Fig. 5. Button plots of steady-state biomass ($\tilde{X}$) versus concentration of P (S_r) in the feed medium in P-limited chemostat cultures of *Chlorella vulgaris*: $\blacksquare$, $D = 0.052 \text{ h}^{-1}$; $\bullet$, $D = 0.107 \text{ h}^{-1}$; $\blacktriangle$, $D = 0.151 \text{ h}^{-1}$; $\triangle$, $D = 0.195 \text{ h}^{-1}$.

Fig. 6. Phosphorus content of biomass (α) as a function of specific growth rate (μ) in steady states of chemostat cultures of *Chlorella vulgaris*: $\bullet$, with P-limited growth; $\blacktriangle$, with N-limited growth. The dashed line is an extrapolation of the P-limited curve.

Fig. 7. (a) Specific growth rate (μ) and (b) specific rate of P uptake (Q) as functions of substrate P concentration (S) in steady states of P-limited chemostat cultures of *Chlorella vulgaris*. The lines drawn are the theoretical curves of the relations $\mu = \mu_m S^n / (S^n + LK_{sh})$ and $Q = Q_m S^n / (S^n + K_{sh})$, where n , μ_m , Q_m and L have the values given in Table 1; the value of K_{sh} was taken from the Q_P data in Table 1.

of writing in our model $\Delta\alpha = \alpha - \alpha_0$ we should write $\Delta\alpha = \alpha - \alpha_0 - \alpha_s$ where α_s is the stored P. Investigation of how α_s depends on growth rate would require more data, particularly at the high specific growth rates.

DISCUSSION

The experimental data for steady state N-limited growth of *Chlorella vulgaris* indicates that there was a strong homotropic cooperative effect on N (urea) uptake. The ratio $L = \alpha_0/\alpha_m$ for N uptake can be accounted for by starch storage because starch can contribute over half of the total cell dry weight in N-limited cultures of this strain of *C. vulgaris* (Watts Pirt & Pirt, 1977). Hence the deviation of L from unity and the consequent difference between K_s and K'_s values for N uptake can be considered artefacts. Thus the relation of *C. vulgaris* growth rate to N (urea) concentration differs from the Monod relation mainly in the strong cooperativity effect.

The data for P-limited growth indicates a small cooperative effect since the Hill number (n) is 1.3. The large difference between the Hill numbers for N and P uptake indicates variation in the biochemical mechanisms of these two processes.

The main difference between the Monod expression and that observed here for the effect of P concentration on the specific growth rate of *C. vulgaris* lies in the effect of yield variation. The ratio L for P uptake was much smaller (about threefold) than that for N uptake. We have no information about starch production with P-limited growth, but if this occurs, it seems unlikely that it could exceed that obtained with N limitation where the maximum starch content (Watts Pirt & Pirt, 1977) could account for L as low as 0.47. Some increase in α_p with increase in μ would be expected because of increased RNA content. However, the small value of L for P uptake depends much on the large increase in α just about when $\mu = \mu_m$. This sudden increase in α_p could be the result of storage of P as polyphosphate when the culture is nearly saturated with phosphate. The same effect, we have postulated, could account for the discrepancy between the K_{sh} value from the α data and that from the Q data for P uptake. Evidence for a discontinuity in Q_P at the onset of P limitation was presented by Nyholm (1977).

Nyholm (1976, 1977) considered that the specific growth rate is independent of the external concentration of a conserved substrate and proposed instead that μ is determined by the substrate content (α) of the biomass; in fact he termed α the 'internal substrate concentration'. Pursuing this argument, Nyholm (1976) derived the empirical equation $\mu/\mu_m = (\alpha - \alpha_0)/(\alpha_m - \alpha_0)$ and showed that it agreed with data for K or P or Mg limited growth of

bacteria in chemostat cultures. This relation approximates to our equation 10, which may be written as $Q/Q_m = (\alpha - \alpha_0)/(\alpha_m - \alpha_0)$; however $Q/Q_m = \mu/\mu_m$ only if the growth yield ($1/\alpha$) is constant, which is not the case. Our data show that the external substrate concentration can be related to μ and our model re-affirms the μ - S relation and incorporates the yield effect. It should be noted that our model is proposed essentially for steady-state conditions rather than for transient states.

The effects observed have implications of technological and ecological importance. The more rapid approach of μ to its asymptotic value μ_m when there is a cooperative effect means that the organism can maintain practically its maximum growth rate down to lower S values than would otherwise be the case. If the Hill number is large then, at low S values, $d\mu/dS$ is very small and it could appear that S has to exceed a threshold value before growth begins.

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