

Stoichiometric Interpretation of *Escherichia coli* Glucose Catabolism under Various Oxygenation Rates

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Metabolic by-product secretion is commonly observed in oxygen-limited cultures. Oxygen limitations occur because of limits in the capacity of the respiratory system or because of the oxygenation limits of the cultivation method used. The latter restriction is of considerable practical importance since it results in a critical cell concentration above which oxygenation is insufficient, leading to by-product secretion. In this study we used a flux balance approach to determine optimal metabolic performance of *Escherichia coli* under variable oxygen limitations. This method uses linear optimization to find optimal metabolic flux patterns with respect to cell growth. Cell growth was defined as precursor requirements on the basis of a composition analysis. A growth-associated maintenance requirement of 23 mmol of ATP per g of biomass and a non-growth-associated maintenance value of 5.87 mmol of ATP per g (dry weight)-h were incorporated on the basis of a comparison with experimental data. From computations of optimal growth increased oxygen limitations were found to result in the secretion of acetate, formate, and ethanol in that order. Consistent with the experimental data in the literature, by-product secretion rates increased linearly with the growth rate. The computed optimal growth under increasing oxygen limitation revealed four critical growth rates at which changes in the by-product secretion pattern were observed. Concomitant with by-product secretion under oxygen limitations were changes in metabolic pathway utilization. The shifts in metabolism were characterized by changes in the metabolic values (computed as shadow prices) of the various redox carriers. The redox potential was thus identified as a likely trigger that leads to metabolic shifts. Below a critical oxygen limitation of 0.88 mmol/g (dry weight)-h the anaerobic by-products were secreted at all growth rates, thus defining the upper limits of anaerobic growth. The general agreement between the optimal metabolic results derived here and experimental observations may prove to have evolutionary and ecological significance for stoichiometrically optimal metabolism.

Microbial cultures are often oxygen limited. Restrictions in oxygen supply can occur because of either capacity limits of the respiratory system or external supply restrictions. When oxygen is limited, the carbon substrate is only partially oxidized, which leads to by-product secretion. The secretion of metabolic by-products allows the generation of energy while cellular redox metabolism is balanced.

During cell culture the rate of oxygen consumption has to balance with the rate of oxygen supply. Mathematically, this balance is expressed as:

$$q_{O_2} \cdot X_v = k_L a \cdot c^* \quad (1)$$

where q_{O_2} is the oxygen consumption per cell, X_v is the viable cell density, c^* is the dissolved oxygen concentration, and $k_L a$ is the mass transfer coefficient that is a characteristic of the particular cell culture equipment used.

Figure 1 shows graphically the balance between oxygen demand and supply as determined by equation 1. The x axis represents the typical oxygen mass transfer capacity of the equipment, while the oxygen demand of a culture is shown on the y axis. Typically, cultivation is initiated at low cell concentrations, usually in the aerobic region. Cell growth results in increasing oxygen demand, as indicated by the arrow in Fig. 1. At a certain cell concentration the oxygen demand of the culture exceeds the supply capacity, resulting in a partially aerobic culture. In this paper we examine the effect of oxygen limitations on optimal by-product secretion and metabolic behavior.

Recently, stoichiometrically based methods have been developed for the study of optimal metabolic behavior (9, 16, 22, 27–29). This approach utilizes flux balances around metabolic pathways to define a region of stoichiometrically allowable metabolic flux distributions. Within the domain of allowable flux distributions, linear optimization is used to determine the particular flux distribution that leads to optimal metabolic performance, as measured by a stated criterion. This approach has provided information about the stoichiometric constraints on metabolism and has allowed the determination of fundamental quantities of metabolic physiology, such as limits on catabolic performance and the ability to produce metabolic cofactors (27, 28).

Stoichiometric analysis is well suited to address questions associated with the optimal secretion pattern of metabolic by-products under oxygen limitations. In this study the answers to these questions were obtained for *Escherichia coli* on the basis of the principle of stoichiometric optimality. By-product secretion patterns due to the enzymatic limits of the respiratory chain were evaluated on the basis of stoichiometric optimality. We have also examined the effect of externally imposed oxygen limitations from completely anaerobic to aerobic conditions.

MATERIALS AND METHODS

Stoichiometric analysis. A metabolic steady state was considered for analysis, where metabolic fluxes leading to the formation and degradation of a metabolite must balance, leading to the flux balance equation (9, 22):

$$S \cdot v = b \quad (2)$$

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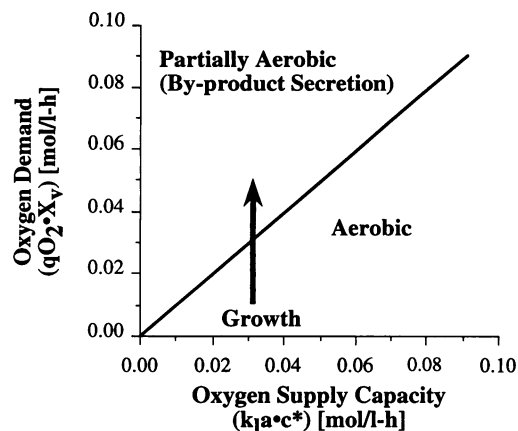


FIG. 1. Oxygen demand of a cell culture in contrast to the supply rate. A typical cell culture apparatus uses surface aeration, agitation, and air sparging depending on the scale of operation. Cell growth results in oxygen demand that outstrips supply capacity, resulting in by-product secretion.

where S is a matrix containing the stoichiometry of the catabolic reactions, v is a vector of metabolic reaction rates, and b is a vector containing the net metabolite uptake by the cell. Equation 2 is typically underdetermined since the number of fluxes normally exceeds the number of metabolites. Thus, a plurality of solutions exists, and a particular solution may be found by using linear optimization by stating an objective and seeking its maximal value within the stoichiometrically defined domain.

Objective. A maximal growth objective (28) was used in this study:

$$\text{minimize } Z = -V_{\text{gro}} \quad (3)$$

$$\sum_{\text{All } M} d_M \cdot M \xrightarrow{V_{\text{gro}}} \text{biomass} \quad (4)$$

where d_M is the requirement (in millimoles per gram of biomass) of the M biosynthetic precursors and cofactors for biomass production and Z is the objective. These metabolic requirements for growth are based on biomass composition (13). V_{gro} is the growth flux, which with an appropriate choice of basis reduces to the growth rate.

Shadow prices. A useful quantity, called the shadow price (γ_i), facilitates the interpretation of the results of linear optimization. It is computed from the mathematical duality of the linear optimization problem (18). Mathematically, the shadow price measures the marginal increase in objective due to an incremental amount of a metabolite provided:

$$\gamma_i = \delta Z / \delta b_i \quad (5)$$

Thus, the shadow price measures the increase in objective possible due to the addition of the i th metabolite. In this study, in which growth was the objective, the shadow price indicates the marginal usefulness of a metabolite toward increasing the growth rate.

The shadow price is shown schematically in Fig. 2. In this example the growth rate increases in a piece-wise linear manner as the oxygen supply increases. At a particular oxygen supply rate the shadow price for oxygen indicates the marginal or local increase in growth rate that can be

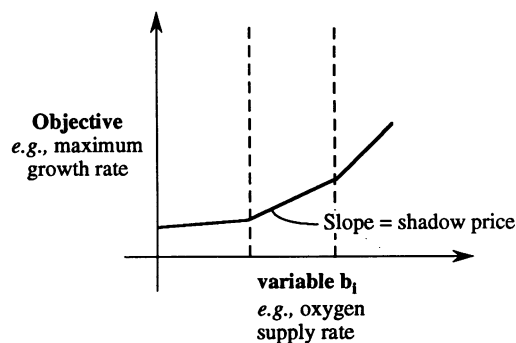


FIG. 2. Schematic representation of the shadow price, which effectively provides the increase in objective possible by providing additional quantities of the i th metabolite or nutrient.

achieved by the addition of oxygen. Thus, the shadow price indicates the utility or usefulness of oxygen in accelerating the growth rate.

Computations. The solutions presented below were computed with Apollo 3500 work-stations. A simplex algorithm implementation technique was used for linear optimization (11).

Metabolic network. The metabolic system is described by the matrix S . The catabolic network used for this study has been described previously (27). In this analysis, by-product formation was allowed from the catabolic network by the incorporation of secretion pathways for acetate, ethanol, formate, lactate, and succinate.

Basis. To introduce uptake and growth rates into the flux balance analysis, we need to introduce a basis. As a basis, consider a certain mass of cells in culture over some period of time such that the product of cell mass and time is 1 g (dry weight)-h. The basis value of 1 g (dry weight)-h may represent 1 g of cells over a period of 1 h or 10 g of cells over a period of 6 min, etc. The unit is analogous to units such as man-hours and kilowatt-hours. This choice of basis allows all fluxes within the metabolic network to be expressed as millimoles per gram (dry weight)-hour. Thus, uptake limits can be specified in the metabolic network and hence define the metabolic capacity. Growth of the bacterium has been defined as a flux draining biosynthetic precursors and cofactors in an appropriate ratio (28). In the context of our choice of basis, the growth flux became grams of biomass generated per gram (dry weight)-hour, which is the growth rate.

Uptake limits. The maximum oxygen uptake rate of *E. coli* cells growing on a variety of carbon sources in aerobic cultures was reported to be about 20 mmol of O_2 per g (dry weight)-h (1). Since a surplus of nutrients was provided in this study, one may consider this limit to represent the maximal respiratory capacity of *E. coli*. Saturation-type kinetic expressions of aerobic glucose consumption have been applied by several workers (7, 14, 24), and the maximum uptake was approximately 20 mmol of Glc per g (dry weight)-h. However, this limit can be exceeded under several growth conditions, such as anaerobiosis. Although the maximum uptake rates described above may vary for different strains as well as for different conditions of growth, for flux balance analysis it is only necessary to recognize that some upper limit on uptake exists.

Maintenance requirements. We now account for both growth-associated maintenance and non-growth-associated maintenance (17, 19–21, 26). Substrate utilization for maintenance and growth is mathematically expressed as:

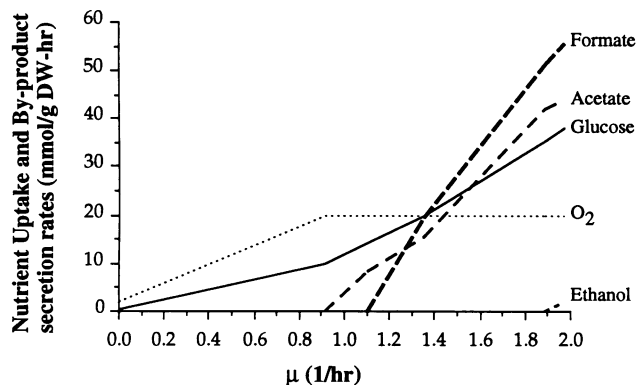


FIG. 3. Optimal aerobic growth and by-product secretion of *E. coli* when glucose is used as a substrate. A maximal oxygen uptake rate of 20 mmol of O_2 per (dry weight)-h was used for the computations. DW, dry weight.

$$q = m_s + (1/Y_{\max}) \times \mu \quad (6)$$

where m_s is the substrate requirement for non-growth-associated maintenance activities, Y_{\max} is the maximum biomass yield that includes growth-associated maintenance, and μ is the growth rate. At 30°C under aerobic conditions, m_s has been observed to have values in the range from 0.055 to 0.07 g of Glc per g (dry weight)-h (17, 20, 23). The parameter Y_{\max} represents the maximum possible yield and has a value of 0.524 g (dry weight) per g of glucose (23). These parameter values have been determined at 30°C, and temperature has been found to have a significant impact on the maintenance requirement (8, 15).

For the analysis described in this study non-growth-associated maintenance requirements are defined as an equivalent ATP maintenance demand in millimoles of ATP per (dry weight)-h. Growth-associated maintenance requirements are defined as an additional ATP requirement per unit of biomass produced. These ATP requirements can be determined from a plot of glucose uptake versus optimal computed growth rate, given the experimentally determined values for m_s and Y_{\max} . Figure 3 shows glucose uptake as a function of the computed optimal growth rate. A linear relationship showing agreement between equation 6 and the flux balance approach was obtained. The corresponding growth-associated ATP requirements are 23 mmol of ATP per g (dry weight), while the non-growth-associated maintenance requirements are 5.87 mmol of ATP per g (dry weight)-h. In the metabolic network the growth-associated ATP requirements are included along with the metabolic requirements for growth, while non-growth-associated maintenance is a drain flux of 5.87 mmol on the ATP pool since the basis corresponds to 1 g (dry weight)-h. The optimal biomass yield has a moderate logarithmic sensitivity of 0.2 to the ATP requirements specified for the flux balance analysis (28).

RESULTS

Growth as a function of the glucose supply. We used linear optimization techniques along with the stoichiometric matrix as explained above to compute the optimal growth rates. The computed optimal growth rate of *E. coli* on glucose increases linearly with glucose utilization up to a growth rate of 0.9 h^{-1} (Fig. 3), when oxygen uptake hits the respiratory constraint

TABLE 1. By-product secretion exhibits a linear relationship with growth rate^a

By-product	μ_c (h^{-1})	v_1 (g/g [dry wt])
Acetate	0.91	2.6
Formate	1.11	3.7
Ethanol	1.89	1.1

^a See equation 7. Secretion parameters were determined by using the maximum aerobic oxygen uptake value of 20 mmol of O_2 per g (dry weight)-h.

of 20 mmol of O_2 per g (dry weight)-h (1). At higher growth rates the oxygen supply is limiting, and glucose cannot be fully oxidized to CO_2 . By-product secretion is observed as a means of eliminating surplus redox from the extra glucose. Acetate is the first by-product secreted as oxygen becomes limiting. At slightly higher growth rates formate is also secreted (Fig. 3). At very high glucose uptake rates ethanol is also secreted in the optimal growth solution.

The computations show a piece-wise linear relationship between by-product secretion and growth rate (Fig. 3). This relationship can be expressed in mathematical form as:

$$v = \begin{cases} 0 & \text{if } \mu < \mu_c \\ v_1(\mu - \mu_c) & \text{if } \mu > \mu_c \end{cases} \quad (7)$$

where v is the by-product formation rate in units of grams of by-product per gram (dry weight)-hour. The values for the parameters in equation 7 for the various by-products are shown in Table 1. The values in Table 1 correspond to a maximum oxygen uptake rate of 20 mmol of O_2 per g (dry weight)-h. An inability to supply this oxygen requirement to the culture would result in acetate secretion at lower growth rates, resulting in different parameter values, as discussed below.

Linear relationships between acetate secretion and growth rate have been observed under a variety of experimental conditions (2, 5, 10). The values reported for the two parameters vary; the μ_c values vary from 0.14 to 0.52 h^{-1} , and the v_1 values vary from 0.645 to 1.6 g of acetate per g (dry weight) (2, 10, 16). The variety of parameter values is perhaps an indication of different oxygen uptake limitations, as discussed below.

The sequence of optimal by-product secretion can perhaps be best explained by considering the energy and redox value of the by-products. Table 2 shows the energy value per unit of redox for various by-products. Acetate has the lowest energy-to-redox ratio and should therefore be secreted solely on the basis of this criterion during oxygen limitation. As oxygen limitation becomes more severe, we also have to consider the redox balance. Formate secretion during the degradation of pyruvate to acetyl coenzyme A is a direct and simple means for eliminating surplus redox. Table 2 also shows the energy and redox generated while the by-products are produced. Although acetate secretion provides the maximum energy from substrate level phosphorylation, it also generates surplus redox. Ethanol and succinate are the only by-products that provide a suitable redox sink. While ethanol secretion generates net energy, succinate secretion requires energy. It is therefore not surprising that in the optimal growth solution ethanol secretion is used as a means for eliminating surplus redox.

Growth on glucose with oxygen limitations. We used linear optimization to determine the optimal growth rates with different oxygen supply rates, varying from completely

TABLE 2. Aerobic energy values for different by-products per unit of oxygen consumed; also listed are the energy and redox generated by anaerobic catabolism of glucose into the by-products

By-product	Energy content (mol) per mol of by-product		Ratio of ATP to O ₂	Anaerobic production (mol/mol of glucose) of:	
	ATP	O ₂		ATP	NADH ^a
Acetate	4.67	2	2.33	4.00	2.00
Formate	1.33	0.5	2.67		
Ethanol	8.33	3	2.78	2.00	-2.00
Lactate	8.33	3	2.78	2.00	0.00
Succinate	9.00	3.5	2.57	-2.33	-2.00
Glucose	18.67	6.0	3.11	0.00	0.00

^a Production of the by-products acetate and ethanol was accompanied by the concomitant production of 2 mol of formate per mol of glucose.

anaerobic to fully aerobic conditions. The optimal growth rate was computed to be a piece-wise linearly increasing function with increasing oxygen uptake rate (Fig. 4). A glucose uptake rate of 10 mmol of Glc per g (dry weight)-h was arbitrarily chosen for these computations; however, the results are consistent with other glucose uptake rates, as described below. There are four breakpoints in the curve, which divide metabolic behavior into five phases. Table 3 shows the shadow prices for several metabolites in the five phases. Below we discuss the five phases individually and discuss changes in metabolic behavior.

(i) **Phase I.** Under completely anaerobic conditions an optimal growth rate of 0.26 h⁻¹ was obtained, assuming a glucose uptake rate of 10 mmol of Glc per g (dry weight)-h,

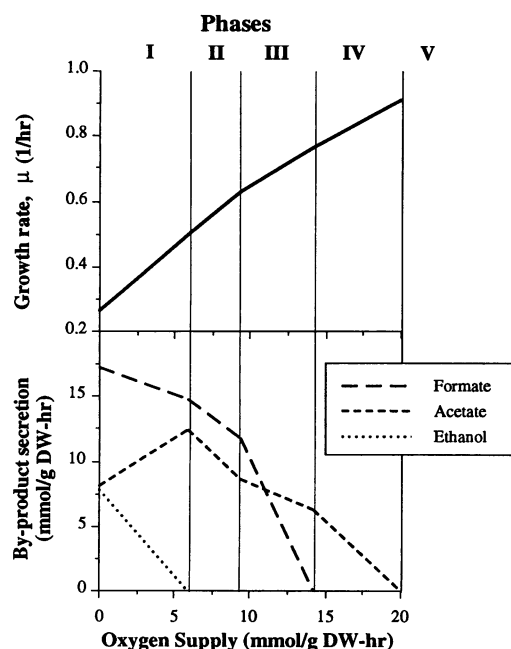


FIG. 4. Optimal by-product secretion and growth for the complete range of oxygenation rates. Five distinct phases of oxygenation were identified on the basis of the growth characteristics and by-product secretion patterns. A glucose uptake rate of 10 mmol of Glc per g (dry weight)-h was used for the computations. DW, dry weight.

resulting in a biomass yield of 0.026 g of biomass per mmol of glucose. The flux distribution exhibited the well-accepted anaerobic pattern of pathway utilization, including the use of anaerobic pyruvate formate lyase (Fig. 5a). The non-use of the tricarboxylic acid cycle in the optimal anaerobic solution has also been observed experimentally. We also observed that the majority of the biosynthetic redox potential was produced by transhydrogenation from NADH to NADPH. The three by-products acetate, ethanol, and formate were secreted. Experimentally obtained by-product secretion patterns vary considerably depending on the culture conditions (3, 4). Table 4 shows experimentally observed by-product secretion rates, as well as the optimal by-product secretion pattern along with the corresponding biomass yield. The optimal by-product secretions and the experimental observations were very similar, indicating that anaerobically growing *E. coli* is capable of stoichiometric optimality. The experimentally observed variation in by-product secretion is probably a function of the ecological niche of the organism, as well as a response to specific culture conditions.

An interpretation of optimal metabolic behavior under anaerobic conditions can be obtained by using the shadow prices shown in Table 3. The three secreted by-products (acetate, ethanol, and formate) were found to have a shadow price of zero, indicating that they cannot be used to improve growth. Succinate and lactate have positive shadow prices and are therefore not secreted in the optimal growth solution. The three redox carriers, NADH, FADH, and QH₂, have negative shadow prices, indicating a desire to eliminate surplus redox.

It is interesting, however, that biosynthetic redox, NADPH, is desirable, as indicated by its positive shadow price. In the metabolic network biosynthetic redox can be produced from NADH by transhydrogenation, a process that utilizes the energy of the proton gradient. Since transhydrogenation is indeed used in the optimal solution (Fig. 5a), we found that the shadow price of NADPH was the sum of the shadow prices of NADH and two protons translocated ($2 \cdot H_{exp}$) (equation 8). As the energetic value of NADPH is greater than its redox undesirability, we found that NADPH has a net utility to the cell.

$$\gamma_{NADPH} = \gamma_{NADH} + (2.0 \times \gamma_{H_{exp}})$$

$$0.0018 = -0.0054 + (2.0 \times 0.0036) \quad (8)$$

(ii) **Phase II.** The next phase is typified by the cessation of ethanol secretion (Fig. 4). Acetate and formate are the only by-products secreted. The corresponding flux distribution is shown in Fig. 5b. The cytochromes are used for the terminal transfer of electrons to oxygen. In this phase we observed the contribution of the pentose phosphate pathway to biosynthetic redox generation, although transhydrogenation was still the major pathway for biosynthetic redox generation.

The ATP shadow prices in Table 3 decreased with increasing aerobiosis, indicating that ATP has greater importance under anaerobic conditions. Except for biosynthetic redox potential, other redox potentials are still undesirable for growth, as indicated by their negative shadow prices. Of the secretable products shown in Table 3, we observed that ethanol has value to the cell and therefore is not secreted. It is surprising that ethanol has value to the cell since ethanol secretion provides the means for eliminating NADH, which has negative value to the cell. This observation is explained

TABLE 3. Shadow prices computed by using dual optimization for selected metabolites under different oxygenation conditions^a

Metabolite	Shadow price (g of biomass/mmol of metabolite) at an oxygen uptake rate of:				
	0 mmol/g (dry wt)-h	7 mmol/g (dry wt)-h	12 mmol/g (dry wt)-h	16 mmol/g (dry wt)-h	20.05 mmol/g (dry wt)-h
O ₂	0.0399	0.0370	0.0283	0.0257	0.000
ATP	0.0109	0.0107	0.0106	0.0104	0.0049
H _{exp} ^b	0.0036	0.0036	0.0035	0.0035	0.0016
NADPH	0.0018	0.0029	0.0035	0.0038	0.0079
NADH	-0.0054	-0.0043	0.000	0.0010	0.0065
FADH	-0.0127	-0.0114	-0.0071	-0.0059	0.0033
QH ₂	-0.0127	-0.0114	-0.0071	-0.0059	0.0033
Acetate	0.0000	0.0000	0.0000	0.0000	0.0242
Ethanol	0.0000	0.0021	0.0106	0.0125	0.0422
Formate	0.0000	0.0000	0.0000	0.0010	0.0065
Lactate	0.0054	0.0064	0.0106	0.0125	0.0422
Succinate	0.0109	0.0128	0.0177	0.0170	0.0504

^a Shadow prices measure the marginal importance of a metabolite toward accelerating growth. The values are the shadow prices determined under the oxygenation conditions of Fig. 5. The specific growth rates for oxygen uptakes rates of 0, 7, 12, 16, and 20.05 mmol/g (dry weight)-h were 0.262, 0.538, 0.701, 0.809, and 0.913 h⁻¹, respectively.

^b H_{exp} represents the energy of the transmembrane proton gradient as the translocation (export) of one proton across the membrane.

by considering acetate secretion (producing one ATP molecule) as an alternative to ethanol secretion. Thus, the positive shadow price of ethanol is the sum of the shadow prices of one ATP molecule and two NADH molecules (equation 9). In other words, the usefulness of generating energy from acetate secretion outweighs the usefulness of ethanol secretion to eliminate redox:

$$\gamma_{\text{ethanol}} = \gamma_{\text{ATP}} + (2.0 \times \gamma_{\text{NADH}})$$

$$0.0021 = 0.0107 + (2.0 \times -0.0043) \quad (9)$$

(iii) **Phase III.** At an oxygen supply rate of 12 mmol of O₂ per g (dry weight)-h we observed another phase of oxygenation, although secretion of acetate and formate still occurred. This phase is characterized by an NADH shadow price of zero (Table 3), indicating that the cell is not forced to eliminate NADH. The energy-poor redox carriers FADH and QH₂ are however still undesirable for growth. The corresponding flux distribution in Fig. 5c shows that both pyruvate dehydrogenase and pyruvate formate lyase are utilized for the conversion of pyruvate to acetyl coenzyme A. Thus, the cell deliberately produces NADH in this conversion rather than eliminating redox as formate. We observed the first utilization of the aerobic pyruvate dehydrogenase in this phase. We also found that transhydrogenation is not used for biosynthetic redox generation and almost all of the biosynthetic redox requirements are met by the pentose phosphate pathway.

(iv) **Phase IV.** By increasing the oxygen supply to 16 mmol of O₂ per g (dry weight)-h we observed the aerobic phase. The optimal flux distribution corresponding to this oxygen supply is shown in Fig. 5d. In this phase we observed the utilization of the complete tricarboxylic acid cycle for energy generation. The anaerobic pyruvate formate lyase is not used. Acetate is the only by-product secreted. The corresponding shadow prices shown in Table 3 indicate that acetate, with a shadow price of zero, is the only nondesirable by-product. Formate and NADH have positive utility for growth. The low-energy redox carriers FADH and QH₂ are still undesirable and have negative shadow prices.

(v) **Phase V.** The last phase of oxygenation is the phase of adequate oxygen supply. Figure 5e shows the optimal flux distribution corresponding to an oxygen uptake rate of 20.05 mmol of O₂ per (dry weight)-h. No by-product is formed, and

the entire substrate is utilized for growth. The tricarboxylic acid cycle is the major source of energy through oxidative phosphorylation. Almost equal amounts of biosynthetic redox are produced by isocitrate dehydrogenase and the pentose phosphate pathway. The corresponding shadow prices shown in Table 3 are positive for all of the metabolites, indicating that there are no surpluses. Oxygen has a shadow price of zero, indicating that there is an adequate supply of this element. All redox molecules have positive shadow prices, indicating their utility for growth in proportion to their conversion to energy by the respiratory chain.

Although in the computations of the five oxygenation states described above we used a glucose supply rate of 10 mmol of Glc per g (dry weight)-h, similar results can be obtained for other glucose supply rates. Figure 6 shows the different phases of oxygenation as a function of the glucose supply rate. The oxygenation condition is determined by the combination of the glucose and oxygen supply rates.

Relationship of cell concentration, glucose, and oxygen supply to by-product secretion. We return now to the question raised in the Introduction. Namely, what is the balance between glucose and oxygen supply rates and the cell concentration at which by-product secretion occurs? Oxygenation in typical cell culture equipment is subject to constraints, as determined by the mass transfer coefficient (k_a). On the other hand, glucose supply is a variable that an experimenter can easily control. For a fixed glucose supply, when oxygen demand increases because of cell growth and exceeds the oxygen supply, by-product secretion is observed.

Figure 7 summarizes the relationship of the three quantities (oxygen supply, glucose supply, and biomass concentration). It shows the conditions under which by-product secretion occurs. In general, acetate is the first by-product to be secreted, followed by formate and finally ethanol. At high glucose uptake rates, such as 10 and 20 mmol of Glc per g (dry weight)-h, oxygen consumption is enzymatically limited. Therefore by-product secretion occurs at all oxygen supply constraints, as indicated by the by-product secretion lines merging with the x axis in Fig. 7. To give a specific example, if the glucose supply rate is 10 mmol of Glc per g (dry weight)-h and oxygenation is limited to 0.02 mol/liter per h, then acetate is always secreted, formate is secreted at

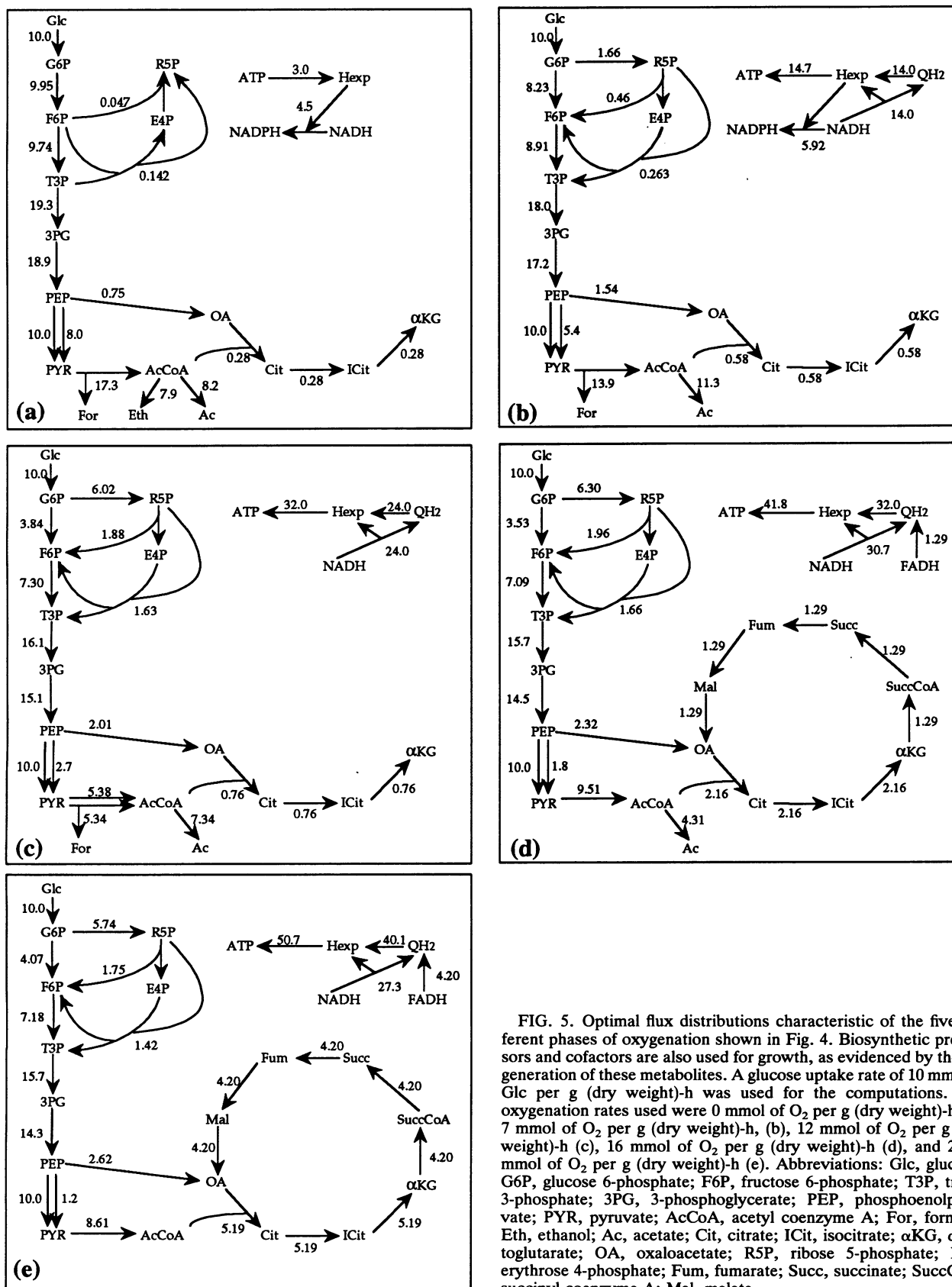


FIG. 5. Optimal flux distributions characteristic of the five different phases of oxygenation shown in Fig. 4. Biosynthetic precursors and cofactors are also used for growth, as evidenced by the net generation of these metabolites. A glucose uptake rate of 10 mmol of Glc per g (dry weight)-h was used for the computations. The oxygenation rates used were 0 mmol of O_2 per g (dry weight)-h (a), 7 mmol of O_2 per g (dry weight)-h (b), 12 mmol of O_2 per g (dry weight)-h (c), 16 mmol of O_2 per g (dry weight)-h (d), and 20.05 mmol of O_2 per g (dry weight)-h (e). Abbreviations: Glc, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; T3P, triose 3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl coenzyme A; For, formate; Eth, ethanol; Ac, acetate; Cit, citrate; ICit, isocitrate; αKG, α-ketoglutarate; OA, oxaloacetate; R5P, ribose 5-phosphate; E4P, erythrose 4-phosphate; Fum, fumarate; Succ, succinate; SuccCoA, succinyl coenzyme A; Mal, malate.

TABLE 4. Comparison of experimental and optimal by-product secretion rates for anaerobic growth of *E. coli* on glucose^a

Product	Secretion rate (mmol/mmol of Glc)	
	Optimal	Exptl ^b
Acetate	0.815	0.75
Ethanol	0.786	0.87
Formate	1.73	1.13
Lactate	0.00	tr
Succinate	0.00	0.12

^a The biomass yields under optimal and experimental conditions were 0.026 and 0.03 g (dry weight) per mmol of Glc, respectively.

^b Data from reference 3.

a cell concentration of 1.5 g (dry weight) per liter, and ethanol is secreted at a cell concentration of 3.5 g (dry weight) per liter.

What are the limits of anaerobic growth? An interesting question concerning the limits of anaerobic growth arises when the results described above are examined. The answer can be found by examining Fig. 3. The computations for Fig. 3 assumed the maximal uptake rate of oxygen, and we observed critical growth rates (μ_c) above which by-product secretion occurred. These critical growth rates did vary with the oxygen uptake rate.

Computation of the critical growth rates with varying oxygen uptake rates demonstrated that there is a linear relationship for the three by-products (Fig. 8). Interestingly, the critical growth rate for ethanol reaches zero at a finite oxygen uptake rate (0.88 mmol of O₂ per g [dry weight]-h). This result means that for any oxygen supply below this oxygen uptake rate all three by-products are secreted. Thus, this condition can be defined as the limit of anaerobic growth.

DISCUSSION

In this study we focused on the optimal performance of the *E. coli* catabolic network based solely on metabolic stoichiometry. Linear optimization was used to locate the metabolic flux distribution that resulted in optimal growth within the set of allowable flux distributions. The results obtained thus reflect a stoichiometric optimality of growth.

With growth defined on the basis of composition-based metabolite requirements, the maintenance requirements were determined from the established relationship between glucose uptake rate and growth rate. We obtained a growth-associated maintenance value of 23 mmol of ATP per g (dry weight) and a non-growth-associated maintenance value of 5.87 mmol of ATP per g (dry weight)-h. These values for maintenance are based on growth data obtained at 30°C and probably vary with temperature (8, 15).

The growth-associated maintenance costs can be compared with the 41.26 mmol of ATP that is stoichiometrically required to synthesize 1 g of biomass from biosynthetic precursors (13). Thus, ATP maintenance requirements for growth are approximately 50% of the amounts required for biosynthesis and polymerization. Under normal growth conditions the non-growth-associated maintenance value of 5.87 mmol of ATP per g (dry weight)-h represents a small fraction of the energy requirements for growth. These ATP maintenance requirements compare well with the previously reported values of 6.8 mmol of ATP per g (dry weight)-h for non-growth-associated maintenance and 71 mmol of ATP per g of biomass for the growth-associated total ATP re-

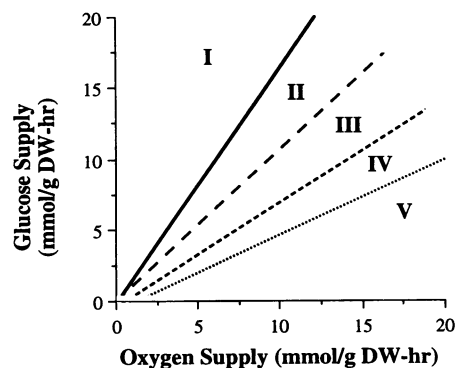


FIG. 6. Five phases of oxygenation shown as a function glucose supply and oxygen supply. A minimum glucose supply of about 0.31 mmol of Glc per g (dry weight)-h is required to fulfill the non-growth-associated maintenance energy requirements. DW, dry weight.

quirement for the anaerobically growing organism *Aerobacter aerogenes* (25).

Using both growth-associated and non-growth-associated maintenance requirements, we can compute the stoichiometrically optimal patterns of metabolic by-product secretion under oxygen limitations. At various levels of glucose uptake we observed optimal secretion of by-products. Secretion is linear with growth rate, occurring above a critical growth rate. Within experimental ranges of cell cultures similar experimental observations for acetate secretion have been reported (2, 5, 10), and the possibility of a restriction in either the respiratory system or a key tricarboxylic acid cycle enzyme has been suggested (16). Increasing the oxygen limitation results in optimal secretion of acetate, formate, and ethanol, in that order. The sequence of secretion is explained on the basis of the energy-to-redox ratios of the by-products, as well as their capability to provide a sink for redox potential.

The critical growth rate determined for the by-products acetate, formate, and ethanol increases linearly with the oxygen supply rate. Above the critical growth rate for ethanol we observed the secretion of all by-products typical of an anaerobic culture. Interestingly, at values below a critical oxygen supply level of 0.88 mmol of O₂ per g (dry weight)-h we observed all by-products secreted at all growth rates. This critical oxygen supply level corresponds to the non-growth-associated maintenance energy requirements of the bacterium.

Oxygenation in a typical cell culture experiment is often limited by mass transfer constraints. Typical mass transfer limits of oxygenation fall in the range from 0.05 to 0.1 mol of O₂ per liter-h. Thus, a growing culture provided with sufficient glucose faces external limits on its oxygen supply. Above a critical cell concentration the oxygen demand of the culture outstrips supply, and by-product secretion results. Therefore, depending on the cell concentration, a glucose-limited culture may face limits on the oxygen mass transfer and thus become oxygen limited. Using the flux balance approach, we were able to predict oxygen limitations and the secretion of by-products.

An optimal growth solution under a variety of oxygenation conditions exhibits several shifts in metabolic behavior. These phases of oxygenation are defined by changes in the values of different redox carriers. Since the redox balance is coupled to several pathways in the metabolic network, we

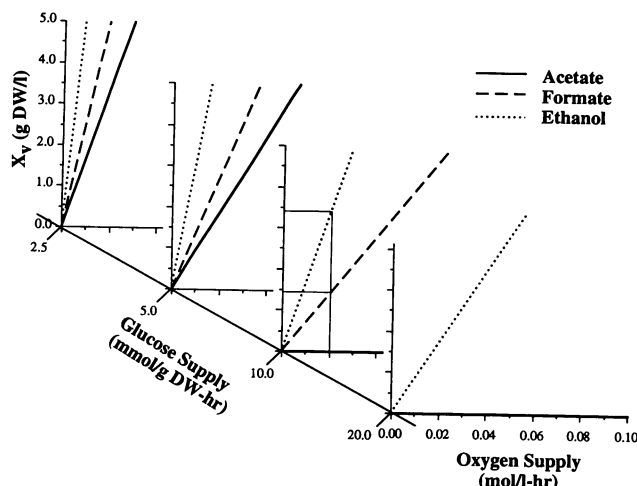


FIG. 7. By-product secretion shown as a function of cell concentration, glucose supply, and oxygen supply. Cell concentrations that exceed the concentrations shown by the by-product secretion lines result in the production of by-product. Fully aerobic cultures may be found only below the acetate secretion line. At high glucose supply rates enzymatic limitations prevent a sufficient oxygen uptake rate, and by-products are secreted at all cell concentrations, as illustrated by the acetate and formate lines merging with the x axis at a glucose level of 20 mmol of Glc per g (dry weight)-h. DW, dry weight.

also observed shifts in metabolic pathway utilization. Several physiological observations are also related to the supply of carbon source and oxygen. In the presence of plentiful glucose, flux redistribution occurs, resulting in the inhibition of oxidative phosphorylation. It is probable that changes in the value of redox carriers, as illustrated by the flux balance approach, are responsible for actuating the regulatory mechanisms that cause the observed changes in pathway utilization. Some effects on metabolic regulation have been observed during the transition from fully aerobic to partially aerobic fermentation at different glucose uptake rates (6, 12). The shifts of metabolic behavior are externally observable in the form of changing by-product secretion patterns.

In conclusion, we found that physiological observations of metabolic behavior are consistent with optimization of growth rate within stoichiometric constraints. We therefore expect the general principle of stoichiometric optimality to

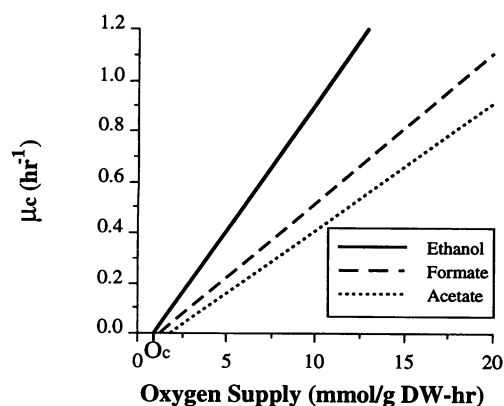


FIG. 8. Variation in the critical growth rate (μ_c) for various by-products at different oxygenation rates. DW, dry weight.

provide a physiological basis for the objectives of metabolic regulatory mechanisms. Stoichiometrically optimal metabolism provides a common basis for the interpretation of observed metabolic by-product secretion patterns under a variety of culture conditions and in the presence of limiting nutrient concentrations.

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