Experimental studies of enzyme kinetics in vitro and metabolic fluxes in vivo have been used by Wright and her colleagues to develop a detailed kinetic model of the tricarboxylic acid cycle in Dictyostelium discoideum. This model has recently been analyzed by two different methods (Albe, K. R., and Wright, B. E. (1992) J. Biol. Chem. 267, 3106-3114; Shiraiishi, F., and Savageau, M. A. (1992) J. Biol. Chem. 267, 22926-22933) in an effort to determine the response of individual fluxes and metabolite concentrations to changes in levels of the enzymes that constitute the system. Individual responses were found to differ significantly in magnitude as well as in sign. Perhaps the most glaring difference concerns the influence of the enzyme succinate dehydrogenase on the flux through the cycle; in one study, it has the maximum influence, whereas, in the other, it has absolutely no influence.

In this paper, we provide a resolution of these discrepancies. We have reconstructed the methodology of Albe and Wright (1) and have been able to reproduce their results in detail. We show that their methodology does not yield a valid steady state analysis, and, consequently, that the conclusions drawn from their analysis must be called into question. First, they concluded that their model is realistic and predictive. It is now clear that their model is ill-determined and has a steady state only for unrealistically narrow conditions. Second, they concluded that their analysis is valid for variations of less than 2% in the levels of the enzymes because they could satisfy summation relationships considered to be mathematically inevitable. It is now clear that these relationships are neither necessary nor sufficient for establishing the validity of an analysis or the appropriateness of a biochemical model. Third, they concluded on the basis of their empirical methodology that certain enzymes are most important in influencing flux through the cycle. It is now clear that these results are inaccurate because of deficiencies in their methodology. Finally, they concluded that steady state analyses cannot be carried out experimentally because of the small variations required in enzyme levels. It is now clear that the requirement for such small variations reflects the ill-determined character of the underlying model and is not a necessary property of the real system.

Albe and Wright (1) have recently performed a steady state analysis of the current working model for the tricarboxylic acid cycle in Dictyostelium discoideum. This model is based on extensive experimental studies of enzyme kinetics in vitro and of metabolite fluxes measured in vivo. Albe and Wright (1) calculated the changes of metabolite concentrations and of fluxes through reactions in response to changes in the levels of the enzymes in the model. If the changes in enzyme levels were less than 2% and the variations were made both above and below the nominal steady state values, then the responses for a given metabolite concentration summed to zero and the responses for a given flux summed to unity, as expected for this model. When the changes in enzyme levels were larger than 2%, or the changes were made unidirectionally either above or below the nominal values, the sums were neither 0 nor 1. From these results, Albe and Wright (1) concluded that a direct experimental test of these summation relationships would be impossible.

The responses of metabolite concentrations to changes in enzyme levels reported in Fig. 4 of the previous paper in this series (2) can be compared directly with those of Albe and Wright (1). There are similarities in that we also require small changes in enzyme levels for the sums to be near 0. However, while they required changes of less than 2%, we could not obtain sums near 0 with changes greater than 0.35%. Furthermore, individual values for the logarithmic gains (control coefficients) in Albe and Wright (1) are often quite different from the empirically determined (and theoretically expected) values in Fig. 4 of Shiraiishi and Savageau (2). The reasons for these discrepancies are not immediately apparent, but there are two likely sources. First, the two studies used different methodologies, and this could be a source of difficulty. Second, it is possible that some source of error has inadvertently been introduced that would then give rise to different results.

In an attempt to resolve these differences, we have reconstructed the methodology of Albe and Wright (1). The rationale is as follows. If one could independently reproduce their results, then the discrepancies would be less likely due to inadvertent error and more likely due to differences in methodology. On the other hand, if one could not reproduce their results with their methodology, then inadvertent error would be likely. The results to be presented in this paper show that the discrepancies mentioned above are due to differences in methodology.
MODEL OF THE TRICARBOXYLIC ACID CYCLE AND ALTERNATIVE SYSTEMIC REPRESENTATIONS

A schematic for the current model of the tricarboxylic acid cycle in Dictyostelium is reproduced in Fig. 1 for easy reference. See the first paper of this series (3) for a full discussion of the terminology and definitions that will be used here. The fundamental equations that characterize the dependent concentrations in this model are given by Kirchhoff's node equations.

\[
\frac{dX_i}{dt} = (v_{i,1} + v_{i,2} + v_{i,3} - v_{i,4}) = (V_{i,1} + V_{i,2} + V_{i,3} - V_{i,4} - V_{i,5})
\]

In these equations, \( v_i \) signifies the rate of utilization of metabolite \( X_i \), for the production of metabolite \( X_{i,1} \). These same reactions also are represented in an alternative notation that is convenient for relating the S-system and Generalized-Mass-Action (GMA) representations within the power-law formalism (4): \( V_{i,k} \) represents the rate of the \( k \)th reaction synthesizing \( X_i \), and \( \bar{V}_{i,k} \) represents the rate of the \( k \)th reaction degrading \( X_i \). The Michaelis-Menten representation of the individual rate laws in Equation 1 is given in Albe and Wright (1). These rate laws are reproduced with a numbering convention appropriate for our analysis in the Appendix of Shiraishi and Savageau (3).

Although, as mentioned above, the previous studies have reported comparable data for metabolite concentrations, they have used alternative methods to report the fluxes. Albe and Wright (1) have reported results for aggregate fluxes through reactions, while we have previously reported results for aggregate fluxes through pools (2). For ease of comparison, in this paper we shall report aggregate fluxes through reactions, which requires only a minor change in the procedures of Shiraishi and Savageau (3). Instead of using the power-law representation of aggregate rate laws for flux through pools, we use the power-law representation of aggregate rate laws for flux through enzyme-catalyzed reactions.

For example, the Michaelis-Menten representation of the rate laws for the two reactions removing succinate from the pool \( X_{11} \) are

\[
V_{11,1} = X_{10}X_{11} \quad \text{(Eq. 2)}
\]

\[
V_{11,2} = X_{16}(X_{11} - 0.100X_{13})/(0.100 + X_{11} + 0.100X_{12}) \quad \text{(Eq. 3)}
\]

where \( V_{11,1} \) and \( V_{11,2} \) represent the rate of loss of \( X_{11} \) by the first and second reaction, respectively. The corresponding power-law representations about the nominal steady state with \( X_{11} \), \( X_{12} \), \( X_{16} \), and \( X_{32} \) values of 0.801, 0.0400, 3.15, and 1.00 are

\[
V_{11,1} = X_{10}X_{11} \quad \text{(Eq. 4)}
\]

\[
V_{11,2} = 3.878X_{10}X_{11}X_{13}^{0.00944} \quad \text{(Eq. 5)}
\]

Obviously, Equation 2 is already in the power-law form. The
parameters in Equation 5 can be obtained from Equation 3 as follows

\[ h_{11,12} = (\partial V_{-11,2}/\partial X_{11})(X_{11}/V_{-11,2}) = 0.120 \]
\[ h_{11,22} = (\partial V_{-11,2}/\partial X_{11})(X_{11}/V_{-11,2}) = -0.0044 \]  
\[ h_{12,12} = (\partial V_{-12,2}/\partial X_{11})(X_{12}/V_{-12,2}) = 1.00 \]
\[ h_{22,12} = (\partial V_{-22,2}/\partial X_{11})(X_{12}/V_{-22,2}) = 1.00 \]
\[ \beta_{11,2} = [V_{-11,2}(X_{11}^{11,2}X_{12}^{12,2}X_{11}^{11,2}X_{12}^{12,2})] = 0.878 \]

where the subscript “0” indicates that the results are evaluated at the nominal steady state values. These results should be compared with those for the same example given in Shiraishi and Savageau (3).

As indicated above, the parameters in the S-system representation are easily related to those in the GMA representation. The kinetic orders of the S-system representation (3) are simply averages of the corresponding kinetic orders of the GMA representation (Equations 4 and 5) weighted according to the fraction of the total flux passing through each of the reactions. For example,

\[ h_{11,12} = (V_{-11,2}/V_{-11,2})h_{11,12} + (V_{-11,1}/V_{-11,1})h_{11,12} \]
\[ 0.317 = (0.801/3.58)(1) + (2.78/3.58)(0.120) \]
\[ h_{11,22} = (V_{-11,2}/V_{-11,2})h_{11,22} + (V_{-11,2}/V_{-11,2})h_{11,22} \]
\[ -0.00733 = (0.801/3.58)(0) + (2.78/3.58)(-0.0044) \]
\[ h_{12,12} = (V_{-12,2}/V_{-12,2})h_{12,12} + (V_{-12,1}/V_{-12,1})h_{12,12} \]
\[ 0.776 = (0.801/3.58)(0) + (2.78/3.58)(1) \]
\[ h_{12,22} = (V_{-12,2}/V_{-12,2})h_{12,22} + (V_{-12,2}/V_{-12,2})h_{12,22} \]
\[ 0.224 = (0.801/3.58)(1) + (2.78/3.58)(0) \]

The steady state solution for the fluxes in Equation 1 is obtained from the steady state solution for the metabolite concentrations as before, except that the power-law representation of fluxes through reactions rather than fluxes through pools is used.

METHODS

**Determination of Steady State Values—**Logarithmic gains are determined from a knowledge of the steady state values for concentrations and fluxes. Therefore, the method of obtaining these steady state values is crucial. The time derivatives in Equation 1 are 0 in steady state, and the behavior of the system is then completely described by the solution of the resulting nonlinear algebraic equations. In Shiraishi and Savageau (2), we obtained the steady state solution for the S-system representation by analytical means and for the Michaelis-Menten representation by numerical means. These methods were found to be in excellent agreement.

Albe and Wright (1) obtained their steady state results by solving the differential equations in the Michaelis-Menten representation for a fixed period of 10 min. They show the values of the dependent variables remaining largely unchanged after 5 min. We have reconstructed their results by solving the differential equations in the S-system representation (Equations 1 and A1 of Shirai and Savageau (3)) with a precision of 15 significant digits for a 10-min period using the numerical methods previously described (5, 6).

**Theoretical Determination of Logarithmic Gains—**The logarithmic gain in metabolite concentration is given by the ratio of percentage change in a dependent concentration, \( X_i \), to an infinitesimal percentage change in an independent concentration, \( X_j \), while all other independent concentrations and parameters are held constant, and it can be determined by differentiation of the analytical solution for the dependent variable (Equations 2, 2a, and 2b of Shiraishi and Savageau (2)).

\[ L(X_i, X_j) = (\partial X_i/\partial X_j)(X_i/X_j) = (\partial \log X_i/\partial \log X_j) = L_{ij} \]  

(Eq. 8)

This method provides the theoretically expected values.

**Empirical Determination of Logarithmic Gains—**We also have reconstructed the method of finite differences used in Albe and Wright (1) to determine the logarithmic gains. In this method, the system is initially at the nominal steady state. The value for one of the independent variables, \( X_j \), is changed by +2%, and the dynamic response of the system is simulated for a period of 10 min. The same procedure is then repeated for a change of −2%. Finally, the corresponding logarithmic gains are then calculated as follows:

\[ L(X_i, X_j) = (\log X_i^* − \log X_i^(-))/\log X_j^* − \log X_j^- \]  

(Eq. 9)

\[ L(X_i, X_j) = (\log X_i^* − \log X_i^-)/\log X_j^* − \log X_j^- \]  

(Eq. 10)

\[ L(X_i, X_j) = (\log X_i^* − \log X_i^(-))/\log X_j^* − \log X_j^- \]  

(Eq. 11)

where the difference between \( X_i^* \) and \( X_i^- \) is 4%, and the other differences are determined from the values of the two dynamic solutions at 10 min following the change in independent variable.

**RESULTS**

**Theoretically Expected Logarithmic Gains in Fluxes—**The logarithmic gains characterize the propagation of biochemical signals throughout the system (7). These system properties are obtained by a single analytical solution of the steady state equations within the framework of the S-system representation (8). When the methods of the previous section are applied to the current model for the tricarboxylic acid cycle in Dictyostelium, one obtains the results shown in the three-dimensional plot of Fig. 2. The magnitude of a particular logarithmic gain represents the influence of a given independent variable on the flux through a specific reaction. There are seven important patterns revealed by the distribution of influence as depicted in this figure.

First, there are 14 fluxes \( v_{11,12}, v_{12,13}, v_{13,21}, v_{12,50}, v_{21,50}, v_{23,11}, v_{31,10}, v_{10,60}, v_{11,14}, v_{14,11}, v_{11,21}, v_{10,10}, v_{10,12}, v_{12,10}, \) and \( v_{10,12} \), including those through the main portions of the cycle, that are totally unaffected by changes in most independent variables. The logarithmic gains in these 14 rows that have magnitudes less than D = 14, which is 0 within the numerical precision of the computation, are shown as shaded squares. The only way that these 14 fluxes can be altered is by a change in 1 or more of the 6 rate constants \( X_{10}, \) through \( X_{30} \) for the irreversible fluxes bringing carbon into the cycle, or, in some instances, by a change in one of the cofactors \( X_{10}, \) through \( X_{30}, \) which are independent variables in the current model. This is unusual for a system that is so rich in interactions among its component processes and indicates that some feature of the model is severely restricting the ability of the signals to propagate through the system.

Second, the fluxes most influenced by change in the independent variables are those through the reactions alanine transaminase \( v_{10,60}, \) glutamate to succinate \( v_{11,14}, \) and alanine to pyruvate \( v_{11,21}. \) This is seen clearly in the two-dimensional projection on the right, where the magnitudes for a given flux \( v_{10,60} \) have been summed over all the independent variables \( X_j. \) The solid portion of each bar represents the sum of the positive logarithmic gains, whereas the hatched portion represents the sum of the negative logarithmic gains.

Third, the independent variables that have the greatest influence on those fluxes that do respond are the concentrations of malic enzyme \( X_{10}, \) malate dehydrogenase \( X_{10}, \) and...
FIG. 2. Theoretically expected influence of independent variables on fluxes through reactions as determined by the magnitudes of the logarithmic gains. The three-dimensional plot displays the magnitudes of the logarithmic gains as a function of the fluxes $u_k$ and the independent variables $X_i$. Logarithmic gains with value $0$ are shown as shaded squares. The two-dimensional projection on the right gives the magnitudes for a particular flux $u_k$ summed over all independent variables $X_i$. The two-dimensional projection on the left gives the magnitudes for a particular independent variable $X_i$ summed over all the fluxes $u_k$. The solid bars in each projection represent the sum of positive logarithmic gains, and the hatched bars represent the sum of negative logarithmic gains. Logarithmic gains for fluxes through reactions were calculated analytically using Equation 9. See text for discussion.

NAD [$X_{30}$]. This can be seen in the two-dimensional projection on the left, where the magnitudes for a given independent variable are summed over all the fluxes, and, again, contributions of positive and negative logarithmic gains are shown separately. These enzymes are all closely associated with the metabolism of pyruvate. The large and inverse nature of the logarithmic gains with respect to changes in $X_{18}$ and $X_{19}$ shows that the distribution of flux at the malate branch point is critical. For example, an infinitesimal increase in the concentration of malic enzyme [$X_{39}$] results in an increase for the flux through alanine transaminase [$u_{21}$] that is 83-fold greater than the magnitude of the stimulus; whereas an infinitesimal increase in the concentration of malate dehydrogenase [$X_{33}$] results in a decrease in the same flux that is 60-fold greater than the magnitude of the stimulus.

Fourth, there are two independent variables [$X_{30}$ and $X_{31}$] that have absolutely no effect on the fluxes in the model. These variables represent the levels of the two enzymes, aconitase and isocitrate dehydrogenase, that follow the irreversible synthesis of citrate. The two columns of zero logarithmic gains with respect to changes in these independent variables also are shown as shaded squares in Fig. 2. This behavior is easily understood: these two enzymes are downstream in an unbranched pathway with a constant influx (2), changes in their concentrations can have no effect on the flux through the pathway, and, in this model, there is no other way for them to influence the rest of the system.

Fifth, if one sums the signed logarithmic gains with respect to the enzyme concentrations [$X_{14}$ through $X_{39}$], one finds that the value is unity for each flux (data not shown), as is to be expected for the special case of a model that is homogeneous of degree one in the enzyme concentrations. There are negative as well as positive values in each of these sums, and, as was pointed out above, the negative values associated with branches can be much larger than unity. Hence, an increase in the influence exerted by one enzyme does not imply that there must be a compensatory decrease in the influence of one or more other enzymes. The appropriate measure of total influence and its distribution over the enzymes is provided by the sum of the magnitudes of the logarithmic gains. If one sums the magnitudes of the logarithmic gains with respect to the enzyme concentrations [$X_{14}$ through $X_{39}$], one finds that the values for the fluxes exhibit a wide range, from 1 to 264 (data not shown). This demonstrates that the total influence is not limited by some small conserved quantity and can be much greater than unity.

Sixth, it can be seen from Fig. 2 that independent variables other than enzyme concentration (i.e. $X_{30}$ through $X_{31}$) have a strong influence on the behavior of the system. For example, the high logarithmic gains with respect to changes in NAD [$X_{31}$] suggest that if the cofactor concentrations were allowed to vary, as they do in the real system, signal propagation through the system would be more realistic. Thus, any complete assessment of system behavior must include an exami-
nation of all the independent variables.

Finally, the fluxes from protein to specific intermediary metabolites are not influenced, except by changes in the levels of the corresponding enzymes, because these reactions are assumed to be irreversible in the current model.

In summary, the logarithmic gains in fluxes through reactions provide the same general picture as the previously reported logarithmic gains in fluxes through pools (2), but in an alternate form.

Theoretically Expected Logarithmic Gains in Metabolite Concentrations—The logarithmic gains in metabolite concentrations are exactly the same as those reported earlier (2); these are repeated in Fig. 3 for ease of comparison with the results obtained by the method of Albe and Wright (1).

Empirically Determined Logarithmic Gains in Fluxes—The logarithmic gains in fluxes through reactions have been determined empirically from finite differences in independent variables, as described under “Methods.” The results are given in the three-dimensional plot of Fig. 4. The pattern of the results according to this method is quite different from that observed in Fig. 2.

First, all of the dependent fluxes are influenced by changes in all of the independent variables. (The obvious exceptions are the fluxes of protein catabolism, which in this model are considered to be irreversible and dependent only on the levels of the enzymes that catalyze these reactions.) These results are in conflict with the fact that flux through most of the reactions in the cycle is fixed in the steady state and cannot change (2).

Second, the fluxes most influenced by change in the independent variables are those through the reactions alanine transaminase \( [v_{ahs}] \), aspartate transaminase \( [v_{as}] \), Asp \( \rightarrow \) oxalacetate 1 \( [v_{as}] \), and Asp \( \rightarrow \) oxalacetate 2 \( [v_{as}] \). This is seen in the two-dimensional projection on the right, where the magnitudes for a given flux \( v_{as} \) have been summed over all the independent variables \( X_i \). Again, the pattern is very different from the theoretically expected pattern in Fig. 2. Although the flux through alanine transaminase is still the most influenced, this influence is an order of magnitude less in Fig. 4.

Third, the independent variables that have the greatest influence on the dependent fluxes are the concentrations of succinate dehydrogenase \( [X_{sd}] \), malic enzyme \( [X_{me}] \), and malate dehydrogenase \( [X_{md}] \). This can be seen in the two-dimensional projection on the left, where the magnitudes for a given independent variable are summed over all the fluxes. There is still an inverse relationship between the logarithmic gains with respect to changes in \( X_{sd} \) and \( X_{me} \). For example, an infinitesimal increase in the concentration of malic enzyme \( [X_{me}] \) results in an increase for the flux through alanine transaminase \( [v_{ahs}] \) that is 4.64-fold greater than the magnitude of the stimulus; whereas an infinitesimal increase in the

Fig. 3. Theoretically expected influence of independent variables on metabolite concentrations as determined by the magnitudes of the logarithmic gains. The three-dimensional plot displays the magnitudes of the logarithmic gains as a function of the dependent metabolite concentrations \( X_j \) and the independent variables \( X_i \). Logarithmic gains with value 0 are shown as shaded squares. The two-dimensional projection on the right gives the magnitudes for a particular metabolite concentration \( X_j \) summed over all independent variables \( X_i \). The two-dimensional projection on the left gives the magnitudes for a particular independent variable \( X_i \) summed over all the metabolite concentrations \( X_j \). The solid and hatched bars in each projection represent the sum of positive and negative logarithmic gains, respectively. Logarithmic gains for metabolite concentrations were calculated analytically using Equation 8. See text for discussion.
FIG. 4. Empirically determined influence of independent variables on fluxes through reactions as determined by the magnitudes of the logarithmic gains. The three-dimensional plot displays the magnitudes of the logarithmic gains as a function of the fluxes $v_{ik}$ and the independent variables $X_j$. Logarithmic gains with value 0 are shown as shaded squares. The two-dimensional projection on the right gives the magnitudes for a particular flux $v_{ik}$ summed over all independent variables $X_j$. The two-dimensional projection on the left gives the magnitudes for a particular independent variable $X_j$ summed over all the fluxes $v_{ik}$. The solid bars in each projection represent the sum of positive logarithmic gains, and the hatched bars represent the sum of negative logarithmic gains. Logarithmic gains for fluxes through reactions were empirically determined using Equation 11. See text for discussion.

concentration of malate dehydrogenase $[X_{10}]$ results in a decrease in the same flux that is 3.41-fold greater than the magnitude of the stimulus. However, when compared to the theoretically expected results (83-fold and 60-fold, respectively), the magnitudes are relatively small, which underestimates the importance of the distribution of flux at the malate branch point.

Fourth, the two independent variables $[X_{24}$ and $X_{26}$] that represent aconitase and isocitrate dehydrogenase, the two enzymes that follow the irreversible synthesis of citrate, have a small but non-zero influence on the fluxes of the system. Again, this is contrary to the theoretical expectation for irreversible reactions in an unbranched pathway with constant influx, which should have absolutely no effect on these fluxes (2).

Fifth, if one sums the signed logarithmic gains with respect to the enzyme concentrations $[X_{14}$ through $X_{30}]$, one finds that the value is unity for each flux (data not shown), as is to be expected for the special case of a model that is homogeneous of degree one in the enzyme concentrations. However, the individual values are all different from the theoretically expected values given in Fig. 2. Indeed, some have opposite sign, which signifies that the responses predicted by the two methods are diametrically opposed. For example, an increase in the level of the enzyme succinate dehydrogenase $[X_{16}]$ would cause a 3.07-fold decrease in the flux from oxalacetate to aspartate $[v_{27}]$ according to theoretical expectation, whereas it would cause a 0.974-fold increase according to the empirical determination.

Sixth, it can be seen from Fig. 4 that independent variables other than enzyme concentration (i.e. $X_{10}$ through $X_{14}$) have a significant influence on the behavior of the system. However, this influence has a very different distribution, and the individual values are generally much less than the theoretically expected values given in Fig. 2.

Finally, the fluxes from protein to specific intermediary metabolites are not influenced, except by changes in the levels of the corresponding enzymes. These results are in agreement with theoretical expectations.

**Empirically Determined Logarithmic Gains in Metabolite Concentrations**—The logarithmic gains in metabolite concentrations have been determined empirically from finite differences in independent variables, as described under "Methods." The results are given in the three-dimensional plot of Fig. 5. Again, there are major differences from the theoretically expected patterns.

First, the concentrations of isocitrate $[X_4]$ and citrate $[X_3]$ are now influenced significantly by changes in a number of enzyme concentrations. Since these metabolites precede irreversible steps in the unbranched portion of the cycle with constant influx, their concentrations should remain unaffected by these changes, except in the case of isocitrate dehydrogenase and aconitase (2).

Second, the metabolite concentrations most influenced by...
FIG. 5. Empirically determined influence of independent variables on metabolite concentrations as determined by the magnitudes of the logarithmic gains. The three-dimensional plot displays the magnitudes of the logarithmic gains as a function of the dependent metabolite concentrations $X_i$ and the independent variables $X_j$. Logarithmic gains with value 0 are shown as shaded squares. The two-dimensional projection on the right gives the magnitudes for a particular metabolite concentration $X_i$ summed over all independent variables $X_j$. The two-dimensional projection on the left gives the magnitudes for a particular independent variable $X_j$ summed over all the metabolite concentrations $X_i$. The solid and hatched bars in each projection represent the sum of positive and negative logarithmic gains, respectively. Logarithmic gains for metabolite concentrations were empirically determined using Equation 10. See text for discussion.

change in the independent variables are those for pyruvate [$X_5$] and acetyl-CoA [$X_6$]. This is clear from the two-dimensional projection on the right, where the magnitudes for a given metabolite concentration $X_i$ have been summed over all the independent variables $X_j$. These are the same two metabolite concentrations that are most influenced according to the results in Fig. 3. However, the distribution is otherwise quite different and the maximum values are an order of magnitude smaller than the theoretically expected values.

Third, the independent variables that have the largest influence on metabolite concentrations are the levels of malic enzyme [$X_{18}$], malate dehydrogenase [$X_{19}$], and NAD [$X_{40}$]. This can be seen in the two-dimensional projection on the left, where the magnitudes for a given independent variable are summed over all the metabolite concentrations. The inverse relationship between the logarithmic gains with respect to changes in $X_{18}$ and $X_{19}$ is evident, but, again, the magnitudes are relatively small, thus under-representing the importance of the distribution of flux at the malate branch point. For example, according to the theoretically expected results in Fig. 3, the increase in concentration of pyruvate [$X_5$] is amplified by a factor of 345 over an increase in concentration of malic enzyme [$X_{18}$]; it is amplified by a factor of $-249$ over an increase in concentration of malate dehydrogenase [$X_{19}$]. On the other hand, according to the empirically determined results in Fig. 5, these same logarithmic gain factors are only 14.8 and $-10.9$, an order of magnitude less.

Fourth, the two independent variables $X_{26}$ and $X_{39}$, which represent the concentrations of aconitase and isocitrate dehydrogenase, have small but non-zero effects on the dependent concentrations. In theory, the levels of these enzymes should have absolutely no effect on the concentrations of metabolites other than their substrates, because these enzymes occupy positions within an unbranched pathway with constant influx.

Fifth, if one sums the signed logarithmic gains with respect to the enzyme concentrations [$X_{14}$ through $X_{39}$], one finds that the values are small but far from 0, especially in the cases of acetyl-CoA [$X_1$] and pyruvate [$X_5$] (data not shown), which is contrary to theoretical expectations for a model that is homogeneous of degree zero in the enzyme concentrations (2). Again, when individual empirically determined logarithmic gains are compared with the theoretically expected values in Fig. 3, one finds differences in sign as well as magnitude.

Finally, it can be seen from Fig. 5 that independent variables other than enzyme concentration have a significant influence on the behavior of the system. However, these influences are underestimated in comparison to the theoretically expected values in Fig. 3.

Resolution of Discrepancies—The empirically determined
values in Figs. 4 and 5 are essentially the same as those reported by Albe and Wright (1), which shows that we have accurately reproduced their methodology. The slight discrepancies might be attributed to differences in numerical precision (4 significant digits (1) versus 15 significant digits (5)). Our dynamic solution of the S-system representation rather than the Michaelis-Menten representation also might contribute to these discrepancies. Nevertheless, over the 10-min period of simulation, there is excellent agreement between the two methods.

The temporal response of the metabolite concentrations shows that the system has not reached a steady state within 10 min (Fig. 6). This could have been expected from the eigen values and the temporal responses reported in Shiraishi and Savageau (2, 9). From these earlier results recall that the system reached a quasi-steady state within 10 min, but required another 1300 min to reach a true steady state. This temporal behavior, however, was produced in response to a transitory change in one of the dependent concentrations, and the system returned to its predisturbance steady state.

In Fig. 6, the stimulus is a sustained 2% decrease in the level of malate dehydrogenase, the independent variable \( [X_{in}] \), and, as was shown in Shiraishi and Savageau (2), there is no steady state for the Michaelis-Menten representation of the tricarboxylic acid cycle in Dictyostelium under these conditions. The imbalance in the distribution of flux at the malate branch point leads to an accumulation of pyruvate that eventually causes saturation of the pyruvate dehydrogenase and Alanine transaminase reactions, and the system settles into a fixed pattern with nearly constant values for all of the fluxes and all of the metabolite concentrations except for pyruvate \( [X_{p}] \), which increases linearly with time because of the permanently established imbalance of fluxes into and out of the pyruvate pool (Fig. 7).

The above analysis demonstrates that the discrepancies between our results (2) and those of Albe and Wright (1) can be attributed to the empirical methodology of Albe and Wright, which does not yield a valid steady state analysis.

**DISCUSSION**

It is one of the long-range goals of biochemistry to understand the integrated behavior of complex systems in terms of their underlying molecular determinants. Such understanding requires experimental methods for characterizing the molecular components under appropriate conditions and a mathematical formalism or language in which this information can be accurately represented and efficiently analyzed. Although there is no cellular system for which this mode of understanding currently exists, the work of Wright et al. (10) is an example of efforts being made to address this important issue. They have used experimental studies of enzyme kinetics in vitro and metabolic fluxes in vivo to develop within the Michaelis-Menten framework a detailed kinetic model of the tricarboxylic acid cycle in *D. discoideum*.

Albe and Wright (1) have used computer simulation of this model to examine a theoretical approach based on so-called summation relationships, which is claimed to be generally applicable to any experimental or theoretical system that has a stable steady state and in which enzymatic activities can be changed and systemic responses measured, and to predict logarithmic gains in metabolite concentrations and fluxes that result from small changes in the levels of the various enzymes of the system.

On the basis of their analyses, these authors have come to the following conclusions. (i) Their highly data-based metabolic model of the tricarboxylic acid cycle in *D. discoideum* is realistic and predictive. (ii) With changes of \( \pm 2\% \) in enzyme levels, one obtains a valid steady state analysis that yields both accurate calculations of logarithmic gains and the expected values for the summation relationships, which are considered mathematically inevitable. (iii) Their analysis provides a systematic way to predict rate-controlling points, and, on this basis, they determined that the enzymes exerting maximum influence over flux through the cycle are succinate dehydrogenase, malic enzyme, and malate dehydrogenase. (iv) The summation relationships are not satisfied and this method of analysis breaks down when the levels of enzymes are changed by more than \( 2\% \), which implies that this approach cannot be used to analyze experimental data directly because variations less than \( \pm 2\% \) cannot be introduced or measured with sufficient accuracy.

Our own analysis (2) of the model developed by Wright et al. (10) does not support these conclusions. The material under "Results" demonstrates that these alternative analyses reach different conclusions as a consequence of the methods used for calculating logarithmic gains. The empirical method of Albe and Wright (1) makes \( 2\% \) changes in enzyme levels and uses finite differences obtained by simulating the dynamics of the system for a period of 10 min, which was considered sufficient to establish a steady state. However, a steady state is not established within this period (Fig. 6). Hence, this method does not produce a valid steady state analysis, and
the resulting logarithmic gains are in error (Figs. 2–5).

Resolution of these discrepancies cannot be obtained simply by simulating the dynamics for a longer period of time in the hope of obtaining true steady states. As we have seen in Fig. 7, no steady state solution exists when enzyme levels are changed by 2%. This is the same conclusion we obtained earlier by an alternative method (Fig. 4 of Ref. 2), namely, numerical solution of the steady state equations by the Newton-Raphson method (11). The failure to attain a steady state following such minor modifications indicates that this model is ill-determined, in agreement with the results of the detailed sensitivity analysis presented earlier (9). These data indicate serious qualitative problems with the underlying model of the tricarboxylic acid cycle. Hence, our analysis does not support the first conclusion of Albe and Wright (1) that this model of the tricarboxylic acid cycle in Dictyostelium is realistic and predictive.

The fact that the so-called summation relationships are satisfied in both Figs. 2 and 4 and 3 and 5, even though nearly all the individual values for the logarithmic gains are at odds, shows that these relationships are not sufficient to establish the validity of an analysis. In some cases (e.g. see Sorribas and Savageau (4)), these relationships are not satisfied, even when the steady state analysis is valid, which demonstrates that these relationships also are not necessary to establish the validity of an analysis. Hence, these relationships are neither necessary nor sufficient for establishing the validity of an analysis or the appropriateness of a biochemical model. This is in contrast to the second conclusion of Albe and Wright (1) that satisfaction of the summation relationships is evidence for a valid steady state analysis.

The differences in predicted influence of various enzymes on fluxes and metabolite concentrations, as indicated in Figs. 2–5, are major. Perhaps the most glaring difference concerns the influence of the enzyme succinate dehydrogenase on the flux through the cycle. In our analysis (2), this enzyme has absolutely no influence (see also Fig. 2). This is contrary to the third conclusion of Albe and Wright (1) that, based on their method of prediction (see also Fig. 4), succinate dehydrogenase has the maximum influence.

Our results show that a variation of less than 0.35% in the levels of the enzymes is required for a valid analysis using finite differences. Beyond this level of variation, the underlying model has no steady state. This clearly reflects the ill-determined character of the underlying model. The real system in Dictyostelium is unlikely to be so inflexible, because other evidence suggests that much larger variations are usually possible in real systems (8). These results do not support the fourth conclusion of Albe and Wright (1) that this form of steady state analysis is experimentally impractical because of the small variations required.

These results clearly demonstrate that empirically determined logarithmic gains (control coefficients), despite their apparent simplicity, often cannot distinguish between true steady states and pseudo-steady states. Knowledge of the logarithmic gains can only lead to valid understanding of the influences that operate in a given steady state if this knowledge is embedded within the context of an appropriate theory. The theory that we have used includes analytical tools for determining the existence of a steady state, the stability of a steady state, the logarithmic gains, and parameter sensitivities of the system; it also provides accurate numerical methods for predicting the dynamic behavior (12). These features were crucial for our elucidation of the model developed by Wright et al. (10). It was also crucial that our theory did not rely on the summation relationships that are fundamental to the metabolic control theory that Albe and Wright (1) considered, because it was the satisfaction of these relationships that masked more fundamental problems with the underlying model.

As discussed more fully elsewhere (4, 13), the use of summation relationships has resulted in other erroneous conclusions. Indeed, the claim (14–16) that these relationships and their derivatives are completely general in a biological context has been very misleading. The related claim, that these relationships should be incorporated into all analyses and computer programs as a check against error (17), has compounded the problem by giving this mathematical identity an aura of biological inevitability. Once this mistaken notion is accepted, there is likely to be a strong psychological tendency to make observations fit the expectation.

The results of our analysis provide evidence against the continued claim (14–16) that the summation relationships for flux represent a conservation of influence in the sense that an increase in the influence of some enzyme must be accompanied by a corresponding decrease in the influence of one or more other enzymes in the system. Large negative logarithmic gains can occur in many systems (8, 13, 18–21, Fig. 4 of this paper), and this invalidates the notion that influence is conserved. The existence of large negative values in systems with branches specifically invalidates the claim (15, 22) that negative values generally will be small in branched systems and, thus, that the essential validity of the conservation principal can be preserved. As discussed elsewhere (2, 13, 20), the only meaningful way to evaluate the distribution of influence over the enzymes of a system is to determine all the relevant logarithmic gains and sum their magnitudes as the measure of total influence to be distributed. There is no theory to predict what these totals will be a priori; one can only determine the values empirically in each case.

There are still more fundamental problems with the summation and connectivity relationships of metabolic control theory. The validity of these relationships requires that the systems being analyzed are homogeneous in a mathematical sense (19, 23, 4) as well as a physical sense. This problem does not manifest itself with the Michaelis-Menten model of Wright et al. (10). In fact, all models represented in the Michaelis-Menten formalism will exhibit these relationships, not because they are necessarily appropriate models for real systems, but, because these relationships represent a mathematical identity that is valid for a certain class of mathematical objects that happens to include models within the Michaelis-Menten formalism. This identity holds for any system of equations in which the dependent variables can be expressed as a linear combination of the independent variables. Since all rate laws in the Michaelis-Menten formalism are linear functions of independent enzyme concentrations (24, 25), the identity will always be satisfied. However, satisfying summation and connectivity relationships for a model represented within the Michaelis-Menten formalism need have nothing to do with biochemical reality in vivo, which is often in violation of the assumptions that underlie the Michaelis-Menten formalism (26). This is undoubtedly the reason why Wright et al. (10) find it difficult to reconcile the values of fluxes measured in vivo with the corresponding values deduced on the basis of enzyme kinetic data in vitro. Living systems are in fact extremely heterogeneous (26–28), and the summation and connectivity relationships of metabolic control theory simply do not apply.

The evidence reviewed in the first paper of this series (3) suggests that the power-law formalism provides a more appropriate theoretical framework for the characterization and
analysis of heterogeneous biochemical systems. When the
power-law formalism is applied to the elementary chemical
reactions on which biochemical kinetics is based, one sees
that this formalism includes traditional Mass-Action kinetics
and in turn Michaelis-Menten kinetics as special cases when
the appropriate restrictive assumptions are imposed. It also
has been demonstrated that biochemical systems theory,
which is based on this power-law formalism, applies to het-
erogeneous systems for which the summation and connectiv-
ity relationships of metabolic control theory are invalid (4).
This is consistent with other evidence demonstrating that
biochemical systems theory includes metabolic control theory
as a limited special case.

Thus, one can expect that
the power-law formalism will continue to provide an appro-
priate theoretical framework for the development and under-
standing of realistic models for complex biochemical systems.

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APPENDIX

Power-law Representation of Flux through Reactions

\[ V_{1,1} = V_{-1,1} = X_1 X_3 \]
\[ V_{1,2} = V_{-1,3} = 0.334 X_1^{-0.018} X_5^{-0.742} X_6^{-0.324} \]
\[ V_{1,3} = V_{-1,2} = 1.94 X_1^{-0.036} X_6^{-0.109} X_1^{0.199} X_3^{0.268} \]
\[ V_{2,1} = V_{-2,1} = X_2 X_3 \]
\[ V_{2,2} = V_{-2,2} = X_2 X_3 \]
\[ V_{3,1} = V_{-3,1} = 0.0144 X_3^{-0.049} X_5^{-0.365} X_6^{-0.365} X_3^{-0.365} X_3^{-0.365} \]
\[ V_{3,2} = V_{-3,2} = 0.886 X_3^{-0.012} X_3^{0.012} X_3 \]
\[ V_{2,1} = V_{-2,1} = X_1 X_3 \]
\[ V_{1,2} = V_{-1,2} = X_2 X_3 \]
\[ V_{1,3} = V_{-1,3} = X_1 X_3 \]
\[ V_{4,1} = V_{-4,1} = 0.00482 X_8^{-0.013} X_8^{-0.013} X_8^{-0.013} X_8^{-0.013} X_8^{-0.013} \]
\[ V_{1,2} = V_{-1,2} = 16.2 X_5^{-0.670} X_9^{-0.670} X_9^{-0.670} X_9^{-0.670} X_9^{-0.670} \]
\[ V_{10,2} = V_{-10,2} = 0.152 X_3^{-0.166} X_3^{-0.166} X_3^{-0.166} \]

REFERENCES