Kinetics of Cyclic Enzyme Systems

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(Received May 20, 1965)

SUMMARY

Kinetic rate equations which take into account the effects of depletion of free substrates and free enzymes by binding are derived for a model of cyclic enzyme systems. The reaction velocities predicted from these equations are compared with experimentally observed velocities in three different cyclic enzyme systems, i.e., pyruvic kinase and creatine kinase, pyruvic kinase and hexokinase, and pyruvic kinase and succinic thiokinase. The practical use of the last system for microdetermination of guanine nucleotides in tissue extracts is indicated.

Various applications of these equations are discussed: namely, a simple way to estimate the optimal ratio of enzymes for cycling techniques, a simple criterion for the determination of the rate- limiting enzyme in a cyclic system *in vitro* and *in vivo*, and the possible use for the determination of the molecular weight or the number of active sites per molecule of enzyme.

INTRODUCTION

There are many instances in living tissues where two enzymes share a substrate or cofactor in a cyclic manner. Recently, similar cycling phenomena have been utilized in the techniques developed for the quantitative determination of small amounts of various substrates (1). Although the kinetics of these systems have been briefly described under the different nomenclatures, cyclic system (1; 2, page 208) and regenerative system (3), increasingly wide and frequent applications of the cycling techniques make it desirable to have a thorough and explicit mathematical description of such systems.

In the usual treatment of enzyme kinetics, the assumption is made that the concentration of substrate greatly exceeds that of enzyme. In formulating mathematical expressions for the kinetics of cyclic enzyme systems *in vitro* and *in vivo*, this assumption may not be valid, because such large quantities of enzymes may be employed that the molarities of the enzymes approach or even exceed that of the substrate. Therefore, it has been necessary to formulate a rate equation which takes into account the possible depletion of free substrate by binding with the enzyme. Furthermore, such descriptions may facilitate the understanding of the enzyme systems in the organelles of living cells such as mitochondria where conditions of locally high enzyme and low substrate concentrations may exist.

MATERIALS AND METHODS

GDP, GTP, ADP, ATP, and CoA were obtained from P-L Biochemicals, Inc. DEAE-cellulose (Cellex-D), TPN, DPNH, and phosphoenolpyruvate (PEP) were purchased from the California Corporation for Biochemical Research. Creatine hydrate was purchased from the Fisher Scientific Company. Commercial preparations of nucleotides, except CoA, were purified by ion exchange chromatography, using a DEAE-cellulose column (4, 5).

Pyruvic kinase (PK, rabbit skeletal muscle, type II), lactic dehydrogenase (LDH, beef heart, type III), creatine kinase (CrK, rabbit muscle), hexokinase (HK, yeast, crystalline suspension in ammonium sul-

Mol. Pharmacol. **1**, 178–189 (1965)

fate) and glucose-6-phosphate dehydrogenase (type V) were purchased from the Sigma Chemical Company. All these enzyme suspensions in ammonium sulfate, except creatine kinase, which was a lyophilized powder, were centrifuged; the supernatant fluids were removed; then the enzymes were dissolved in 0.1 M Tris-acetate buffers of appropriate pH containing 1 mg of bovine plasma albumin per milliliter. Succinic thiokinase (STK) was purified from pig hearts according to a procedure improved over that of Cha and Parks (4).

Oxidation or reduction of pyridine nucleotides in reaction mixtures were continuously measured by either a Zeiss model M4QII spectrophotometer, or a Beckman D.U. spectrophotometer, each of which was equipped with a Gilford O.D. converter and a recorder.

DERIVATION OF RATE EQUATION

The simple and realistic system which we wish to consider in this paper may be represented by Eqs. 1 and 2:

$$\mathrm{A} + \mathrm{S} \overset{\mathrm{E}}{\rightleftharpoons} \mathrm{P} + \mathrm{S'} \qquad (1)$$

$$\mathrm{B} + \mathrm{S'} \overset{\mathrm{E'}}{\rightleftharpoons} \mathrm{Q} + \mathrm{S} \qquad (2)$$

where a substrate S is converted to S′ by the first reaction catalyzed by an enzyme E, and S is regenerated by the second reaction catalyzed by E′ in which S′ serves as a substrate. In the present paper, S and S′ will be referred to as the cycling substrates; A and B as noncycling substrates; P and Q as noncycling products.

Unfortunately, the rate equations derived for the similar systems (2, page 209; 3) are so complicated that their practical uses are limited. To avoid such algebraic complexity, the following system (Eqs. 3 and 4) is used as a model:

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \mathrm{S'} \qquad (3)$$

$$\mathrm{E'} + \mathrm{S'} \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} \mathrm{E'S'} \xrightarrow{k'_3} \mathrm{E'} + \mathrm{S} \qquad (4)$$

It is recognized that very few reactions, if any, have a simple mechanism as written above in Eqs. 3 and 4. On the contrary, most reactions involved in a cyclic system are reversible, require more than one substrate, and may have more than one form of enzyme-substrate complex. However, the use of the above simple system as a model to describe far more complex mechanisms can be justified by the following arguments:

1. Regardless of the mechanism, most enzyme reactions follow the Michaelis-Menten type of equation when the concentration of enzyme is reasonably low, e.g.[1]

$$v = V_{\max}\mathrm{S}/(K + \mathrm{S})$$

Therefore, when the following theory is applied to more complex systems, the Michaelis constants and maximal velocities may be replaced, without invalidating the empirically drawn conclusions, by apparent parameters measured at the concentrations of the noncycling substrates (e.g., A and B in Eqs. 1 and 2 above) used in the cyclic system under study.

2. Involvement of isomeric forms of an enzyme-substrate complex does not change the overall form of the rate equation, e.g., $\mathrm{ES} \rightleftharpoons \mathrm{EP}$.

3. In a situation such as the one considered here, the concentrations of the cycling substrate (S and S′) and those of the noncycling products (P and Q) are very low, and the concentrations of other, noncycling, substrates (A and B) are presumably very high, with regard to their respective apparent Michaelis constants. Therefore, the reaction in the reverse direction and product inhibition would be negligible.

In addition to the above-mentioned simplifications, the following assumptions are made:

(a) In the usual treatment of enzyme kinetics, it is customarily and justifiably assumed that the concentration of enzyme is so small compared to that of substrate, that the amount of substrate bound in the enzyme-substrate complex is negligible. However, in the present case, the opposite situation may occur. In other words, if the concentration of enzyme is high, e.g., 0.1 K or greater, and the concentration of sub-

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[1] The customary usage of ( ) or [ ] to designate molar concentrations is omitted in this paper, except where ambiguity may arise.

strate is very low relative to K, only a negligible fraction of the total enzyme is in the form of enzyme-substrate complex, and yet this amount of bound substrate may represent a significant fraction of the total substrate.

(b) Steady states are assumed not only for the individual enzymic reactions, but also for the overall reaction.

In the overall steady state and under these assumptions, the overall reaction velocity (v) is the same as the individual velocities of reactions shown above in Eqs. 3 and 4, which are given by the following equations (Eqs. 5 and 6) as derived in Appendix II.

$$v = \frac{k_3 \mathrm{S}_t \mathrm{E}_t}{\mathrm{S}_t + \mathrm{E}_t + K} \tag{5}$$

$$v = \frac{k'_3 \mathrm{S}'_t \mathrm{E}'_t}{\mathrm{S}'_t + \mathrm{E}'_t + K'} \tag{6}$$

Let the total concentration of the cycling substrate be S_0;

$$\mathrm{S}_0 = \mathrm{S}_t + \mathrm{S}'_t \tag{7}$$

The simultaneous solution of Eqs. 5 through 7 gives the following quadratic equation with respect to v:

$$\begin{aligned} &v^2(\mathrm{S}_0 + \mathrm{E}_t + \mathrm{E}'_t + K + K') \\ &\quad - v\,[k'_3\mathrm{E}'_t(\mathrm{S}_0 + \mathrm{E}_t + K) \\ &\quad + k_3\mathrm{E}_t(\mathrm{S}_0 + \mathrm{E}'_t + K')] \\ &\qquad + k_3k'_3\mathrm{E}_t\mathrm{E}'_t\mathrm{S}_0 = 0 \end{aligned} \tag{8}$$

therefore

$$v = \frac{b - \sqrt{b^2 - 4ac}}{2a} \tag{9}$$

where a and b are the respective coefficients of v^2 and v, and c is the constant in Eq. 8. The negative sign in front of the square root is chosen for the correct solution, because when $\mathrm{S}_0 = 0$, v must be zero, and this is true only when the sign is negative.

A binomial expansion of the square root term in Eq. 9 using the algebraic technique presented in Appendix I gives:

$$v = \frac{1}{\dfrac{\mathrm{S}_0 + \mathrm{E}_t + K}{k_3\mathrm{E}_t\mathrm{S}_0} + \dfrac{\mathrm{S}_0 + \mathrm{E}'_t + K'}{k'_3\mathrm{E}'_t\mathrm{S}_0}} \cdot \left(1 + \sum_{n=1}^{\infty} C_n z^n\right) \tag{10}$$

where n is positive integer numbers, and

$$C_n = \frac{1 \cdot 3 \cdot 5 \cdots (2n-1) \cdot 2^n}{(n+1)!} = 1, 2, 5, \ldots$$

$$z = \frac{k_3k'_3\mathrm{E}_t\mathrm{E}'_t\mathrm{S}_0(\mathrm{S}_0 + \mathrm{E}_t + \mathrm{E}'_t + K + K')}{[k'_3\mathrm{E}'_t(\mathrm{S}_0 + \mathrm{E}_t + K) + k_3\mathrm{E}_t(\mathrm{S}_0 + \mathrm{E}'_t + K')]^2}$$

Equation 10 is the rate equation which includes the effects of depletion of both free substrates and free enzymes by the formation of ES complexes. If $\mathrm{E}_t \ll K$, and $\mathrm{E}'_t \ll K'$, the z terms become negligible compared to 1, and Eq. 10 can be reduced to the counterpart of the classic Michaelis-Menten equation in which the effects of depletion of the free substrates are neglected:

$$v = \frac{1}{\dfrac{\mathrm{S}_0 + K}{k_3\mathrm{E}_t\mathrm{S}_0} + \dfrac{\mathrm{S}_0 + K'}{k'_3\mathrm{E}'_t\mathrm{S}_0}} \tag{11}$$

On the other hand, if $\mathrm{S}_0 \ll K$, and $\mathrm{S}_0 \ll K'$, the z terms also become negligible, and Eq. 10 may be reduced to an equation that takes into account the effects of depletion of the free substrates, but not the free enzyme, by binding:

$$v = \frac{1}{\dfrac{\mathrm{E}_t + K}{k_3\mathrm{E}_t\mathrm{S}_0} + \dfrac{\mathrm{E}'_t + K'}{k'_3\mathrm{E}'_t\mathrm{S}_0}} \tag{12}$$

or

$$\frac{v}{\mathrm{S}_0} = \frac{1}{\dfrac{1}{k_3} + \dfrac{1}{k'_3} + \dfrac{K}{k_3\mathrm{E}_t} + \dfrac{K'}{k'_3\mathrm{E}'_t}} \tag{13}$$

An equation that also takes into account the effect of nonspecific binding is presented in Appendix III.

If $\mathrm{E}_t \ll K$, $\mathrm{E}'_t \ll K'$, $\mathrm{S}_0 \ll K$, and $\mathrm{S}_0 \ll K'$, then Eq. 13 can be further reduced to:[2]

$$\frac{v}{\mathrm{S}_0} = \frac{1}{\dfrac{K}{k_3\mathrm{E}_t} + \dfrac{K'}{k'_3\mathrm{E}'_t}} \tag{14}$$

It should be noted that the quantity v/S_0 has a dimension of reciprocal time, and is the number of cycles per minute. Equation

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[2] Under these conditions, S_t and S'_t can be calculated from $\mathrm{S}_t/\mathrm{S}_0 = a/(a + b)$, and $\mathrm{S}'_t/\mathrm{S}_0 = b/(a + b)$, where $a = K/(k_3\mathrm{E}_t)$ and $b = K'/(k'_3\mathrm{E}'_t)$.

14 clearly indicates that when one of two enzymes (e.g., E') is held at constant concentration, the plot of v/S_0 (the number of cycles per minute) *versus* E_t is a hyperbola, and v/S_0 approaches asymptotically to $k'_3E'_t/K'$ as E_t increases. On the other hand, at finite concentrations of both E_t and E'_t, the two reactions will be mutually rate-limiting.

It is interesting and useful to be able to estimate the optimal ratio of two enzymes, E_t and E'_t. Equation 14 can be converted easily to give the following expression:

$$\text{number of cycles/minute} = \frac{v}{S_0} = \frac{E_0}{\dfrac{K}{k_3\alpha} + \dfrac{K'}{k'_3(1-\alpha)}} \qquad (15)$$

where $E_0 = E_t + E'_t$, $\alpha = E_t/E_0$, and $1 - \alpha = E'_t/E_0$.

Now v/S_0 is considered as a function of α, i.e., $v/S_0 = f(\alpha)$; then Eq. 15 represents a family of curves, one for each value of E_0. The value of α at the maxima of the curves, $\alpha_{\max}$, can be found by solving $d(v/S_0)/d\alpha = 0$; it is independent of E_0 and

$$\frac{\alpha_{\max}}{1 - \alpha_{\max}} = \left(\frac{E_t}{E'_t}\right)_{\text{optimal}} = \sqrt{\frac{K/k_3}{K'/k'_3}} \qquad (16)$$

This relationship holds also for Eq. 14.

Up to this point enzyme concentrations have been expressed as molar concentrations and the first order rate constants, or catalytic center activities, k_3 and k'_3 as moles of substrate converted per minute per mole of ES complex. Since the molecular weights, the catalytic center activities, and the numbers of catalytic centers per molecule of many enzymes are not readily available, the concentrations of enzymes must be expressed in some other convenient units if the above equations are to be of any practical use. This can be done without invalidating the relationships described in Eqs. 11, 13, and 14, provided that the expression is consistent for all terms in an equation. For example, if E_t is expressed as μg/ml of reaction mixture, K should be expressed as μmoles/ml; k_3 as the apparent $V_{\max}$ (μmoles of substrate converted per minute per μg of the enzyme E), instead of the first order rate constant; and α as the weight fraction instead of the molar fraction. Equation 12 should not be used for the computation of v when E_t and E'_t are not expressed as molar concentrations.

EXPERIMENTAL PROCEDURE AND RESULTS

Three different cyclic systems were examined to test if the rate equations derived for the model system describe adequately the more complicated systems. Care was taken to maintain the conditions under which the theory based on the model may be applicable to the more complicated systems: (a) the concentrations of noncycling substrates were kept well above respective Michaelis constants; (b) the noncycling products were not added; (c) the reaction velocities were taken after the establishment of overall steady states.

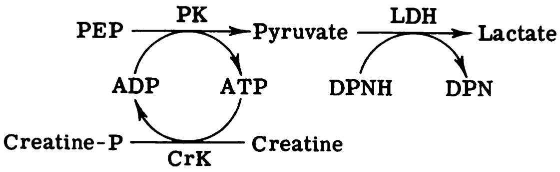
Pyruvic Kinase-Creatine Kinase with ADP and ATP as the Cycling Pair of Substrates

The velocity of interconversion of ADP and ATP was measured as the rate of formation of pyruvate by coupling the system to the lactic dehydrogenase reaction and following the rate of oxidation of DPNH. The reactions involved are schematically illustrated in Scheme 1. The results are presented in Fig. 1. The observed velocities were in reasonably good agreement with those calculated from Eq. 15; however, they were slightly lower than the theoretical values. Nevertheless, these discrepancies are much greater than expected from the contribution by the $(1/k_3 + 1/k'_3)$ term in Eq. 13, and indeed they could be attributed to the incomplete removal of ammonium sulfate from the enzyme preparations.

Despite the slight experimental errors, these data clearly indicate that (a) the velocity is proportional to the sum of two enzymes at a given ratio of the two, as predicted by Eq. 15; (b) the weight fraction of an enzyme to give the fastest velocity at a given sum of the two enzymes can be predicted by Eq. 16.

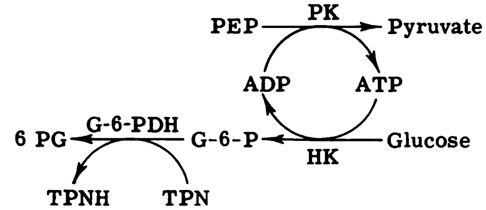
Pyruvic Kinase-Hexokinase System with ADP and ATP as the Cycling Pair of Substrates

The velocity of interconversion of ADP and ATP was measured as the rate of forma-



SCHEME 1

tion of glucose 6-phosphate by coupling the system to the glucose 6-phosphate dehydrogenase reaction and following the rate of reduction of TPN. The reactions in the system are illustrated in Scheme 2. The results are presented in Fig. 2.



SCHEME 2

Pyruvic Kinase-Succinic Thiokinase System with GDP and GTP as the Cycling Pair of Substrates

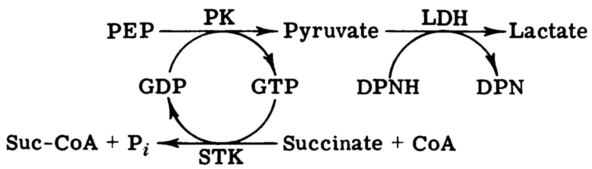
The velocity of interconversion of GDP and GTP was measured by coupling the system to the lactic dehydrogenase reaction as illustrated in Scheme 3. The results are shown in Fig. 3. The discrepancies between the theoretical curve based on Eq. 15 and the experimental points are generally greater than those in Figs. 1 and 2. Perhaps this is largely due to the fact that the concentrations of the cycling substrates (0.0158 mM and 0.0176 mM) were at the Michaelis constant range (0.011 mM), therefore in this case the effect of depletion of free enzyme is seen as in Eq. 11. The principle involved in this cyclic system is being used for the development of micromethods for the determination of guanine nucleotides. The results of a preliminary experiment are presented in Fig. 4. It shows that the same velocity is obtained regardless whether the reaction was initiated with S or S′ (i.e., GDP or GTP). Further details of these methods will be published elsewhere.

From these data, Eq. 14 appears to predict adequately the velocities under the experimental conditions. In all cases, the observed weight fraction of an enzyme at which the velocity of the cycling is maximal agrees very well with those calculated from the simple expression in Eq. 16.

DISCUSSION

The equations developed above for a simple model system appear to describe adequately the *in vitro* behavior of three cyclic systems in which much more complicated enzymic reactions than those in the model system were involved. These equations allow the prediction of the reaction velocities at various concentrations of enzymes and substrates. In addition to such obvious technical applications, it should be reemphasized that, in living tissues, enzymes are often compartmentalized and occur in high local concentration. Thus, in certain cases the equations developed in this paper may more adequately explain the behavior of enzyme systems *in vivo* than do the equations based on the familiar Michaelis-Menten assumptions.

From Eq. 16, one may estimate the



SCHEME 3

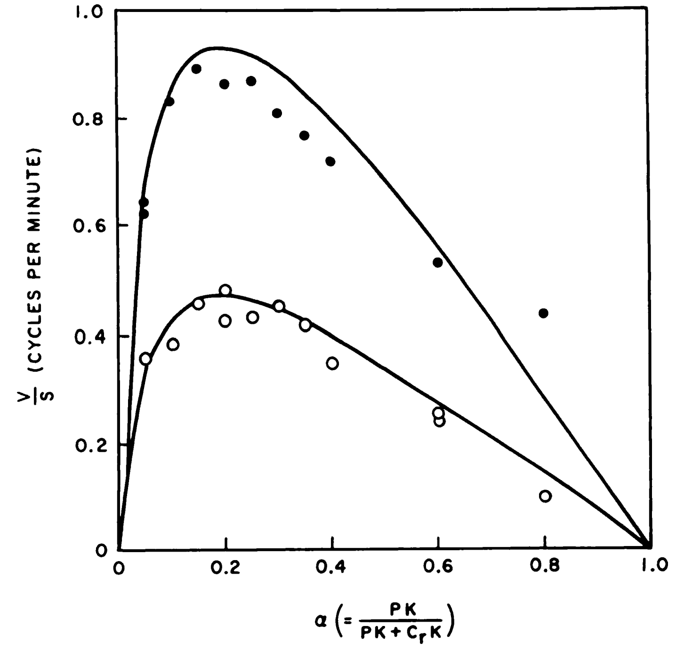


FIG. 1. *Rate of ADP-ATP cycling in pyruvic kinase-creatine kinase system; plot of cycles per minute* (v/S), *versus the weight fraction* $\alpha = PK/(PK + CrK)$

Solid circles (●) represent the experimental points obtained with the total amount of the two enzymes held at 20 μg/ml of reaction mixture; open circles (○) at 10 μg/ml. The reactions were started by adding 4.1×10^{-9} moles of ATP per milliliter of the reaction mixture. The reaction mixture consisted of Tris acetate, pH 8.0, 100 mM; KCl, 100 mM; MgCl$_2$, 10 mM; phosphoenolpyruvate, 1.5 mM; DPNH, 0.2 mM; creatine, 20 mM; lactic dehydrogenase, 10 μg/ml; and appropriate amounts of pyruvic kinase, creatine kinase, and the cycling substrate.

Two theoretical curves were calculated from Eq. 15 employing the parameters determined under similar conditions except the following: (a) for the determination of the apparent Michaelis constant of ADP (K) and the apparent maximal velocity (k_3) for pyruvic kinase, the concentration of ADP was varied with pyruvic kinase held constant at 0.05 μg/ml, and creatine and creatine kinase were not added; (b) for the determinations of the apparent Michaelis constant of ATP (K') and the apparent maximal velocity (k'_3) for creatine kinase, the concentration of ATP was varied with creatine kinase and pyruvic kinase held at 0.1 μg and 5 μg/ml, respectively. K, k_3, K', and k'_3 were 0.294 mM, 0.366 μmole/min/μg, 0.5 mM, and 0.036 μmole/min/μg, respectively. The calculated α_{max} is 0.194.

optimal ratio of the enzymes in any convenient unit of activity. For example, if one of the enzymes of a cyclic system is expensive, or difficult to prepare, the investigator may readily calculate a ratio of enzymes which will achieve the desired results with the minimal expenditure of the more valuable enzyme, simply by expressing k_3 and k'_3 as micromolar unit per unit cost and α as the fraction of the total cost of the enzymes.

In evaluating a cyclic multienzyme system, if one enzyme of the system is in excess of the others, it may erroneously be assumed that it cannot be rate-limiting. The flaw in this assumption for cyclic systems is revealed by Eqs. 15 and 16. For example, in Fig. 3, the portion of the curve to the left of the maximum represents the ratios of pyruvic kinase to succinic thiokinase at which the former is rate-limiting. At the point represented by $\alpha = 0.5$ in the figure, 1 ml of the reaction mixture contained 1.0 μmolar unit of pyruvic kinase and 0.0268 μmolar unit of succinic thiokinase. Therefore, on the basis of relative activities of the two enzymes, it is very easy to conclude that succinic thiokinase was rate-limiting under these conditions, whereas actually the opposite is true.

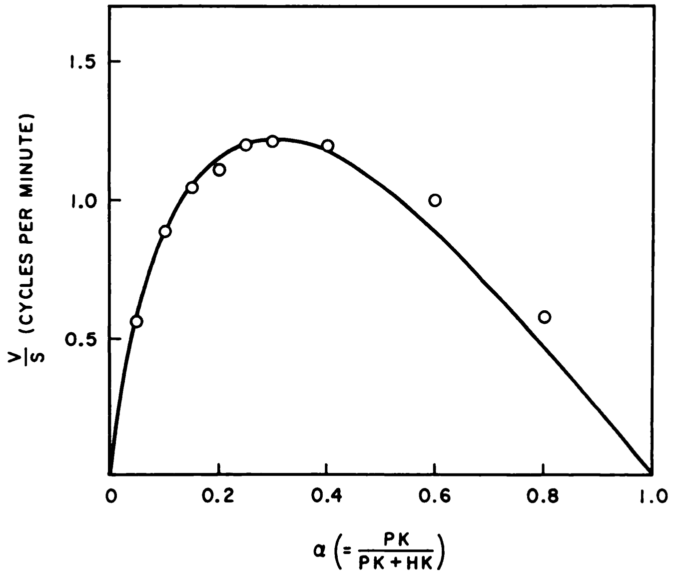


FIG. 2. *Rate of ADP-ATP cycling in pyruvic kinase-hexokinase system; plot of cycles per minute* (v/S), *versus the weight fraction*, $\alpha = PK/(PK + HK)$

Open circles represent the experimental points obtained with the total amount of the two enzymes held at 10 μg/ml of reaction mixture. The reactions were started by adding 6.9×10^{-9} moles of ADP per milliliter of the reaction mixture. The reaction mixture consisted of Tris-acetate, pH 8.0,100 mM; glucose, 5 mM; KCl, 100 mM; $MgCl_2$, 10 mM; phosphoenolpyruvate, 1.5 mM; TPN, 0.1 mM; glucose 6-phosphate dehydrogenase, 0.1 unit/ml; and appropriate amounts of pyruvic kinase and hexokinase.

The theoretical curve was calculated from Eq. 15 employing the parameters determined under similar conditions except the following: (a) for the determinations of the apparent Michaelis constant of ADP (K) and the apparent maximal velocity (k_3) for pyruvic kinase, the concentration of ADP was varied with pyruvic kinase and hexokinase held at 0.02 and 10 μg/ml, respectively; (b) for the determinations of the apparent Michaelis constant of ATP (K') and the apparent maximal velocity (k'_3) for hexokinase, the concentration of ATP was varied with hexokinase held at 0.05 μg/ml, and phosphoenolpyruvate and pyruvic kinase were not added. K, k_3, K', and k'_3 were 0.286 mM, 0.384 μmole/min/μg, 0.5 mM and 0.124 μmole/min/μg, respectively.

It should be noted that, depending on the concentrations of substrates and enzymes relative to respective Michaelis constants, the equations derived above allow one to take into account the effects of depletion of free substrates (Eq. 12 or 13), of free enzymes (Eq. 11), or both (Eq. 10). The kinetics of "mutual depletion systems" has been considered for single-enzyme systems by other workers (3, pages 35 and 66; 6).

Since cycling techniques are used for microdeterminations of various substrates, Eq. 13 deserves particular attention. It indicates that, at a fixed substrate concentration, there is a limit the velocity can reach, no matter how high the concentrations of enzymes. This is due to the depletion of free substrate by binding at the active centers of the enzymes. The effect of binding will be seen even at moderately high concentrations of enzymes. For example, if the molecular weight of an enzyme is 10^5, the Michaelis constant is 10^{-4} M, and the concentration of the substrate is well below the Michaelis constant, then, at an enzyme concentration of 1 mg/ml, 9.1% of the substrate will be tied up; and 50% at 10 mg/ml. On the other hand, it is common knowledge that there is a maximal velocity achievable by increasing the concentration of substrate at a fixed concentration of the enzyme in a single-enzyme reaction. Equation 11 indicates this aspect for the cyclic two-enzyme system.

Equation 13 includes the effect of deple-

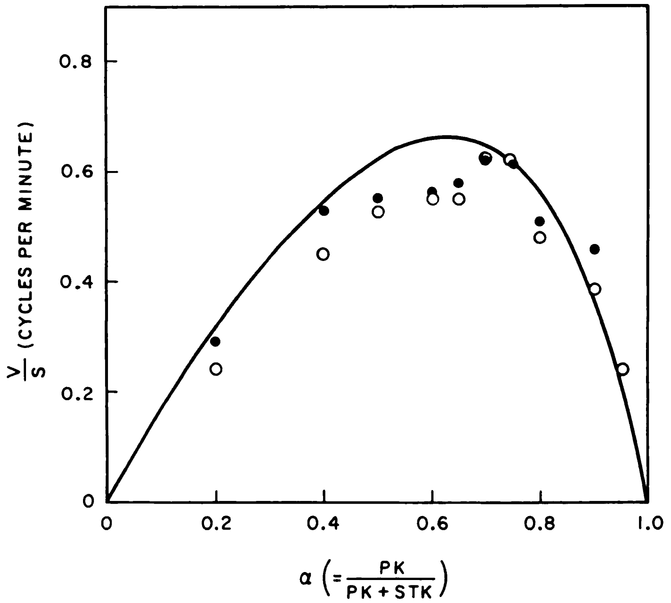


FIG. 3. *Rate of GDP-GTP cycling in pyruvic kinase-succinic thiokinase system; plot of cycles per minute (v/S), versus the weight fraction, $\alpha = PK/(PK + STK)$*

The circles represent the experimental points obtained with the total amount of the two enzymes held at 10 μg, and with 15.8×10^{-9} moles (●) or 17.6×10^{-9} moles (○) of GDP per milliliter of reaction mixture. The reaction mixture consisted of succinate (Tris salt), pH 7.4, 40 mM; CoA, 0.1 mM; KCl, 100 mM; $MgCl_2$, 10 mM; phosphoenolpyruvate, 1.5 mM; DPNH, 0.2 mM; lactic dehydrogenase, 10 μg/ml; and appropriate amounts of pyruvic kinase and succinic thiokinase.

The theoretical curve was calculated from Eq. 15 employing the parameters determined under similar conditions except the following: (a) for the determinations of the apparent Michaelis constant of GDP (K) and the apparent maximal velocity (k_3) for pyruvic kinase, the concentration of GDP was varied with pyruvic kinase held at 0.1 μg/ml, and CoA and succinic thiokinase were not added; (b) for the determinations of the apparent Michaelis constant of GTP (K'), and the apparent maximal velocity (k'_3) for succinic thiokinase, the concentration of GTP was varied with succinic thiokinase and pyruvic kinase held constant at 0.1 and 5 μg/ml, respectively. K, k_3, K', and k'_3 were 1.2 mM, 0.20 μmole/min/μg, 0.011 mM, and 0.00536 μmole/min/μg, respectively.

tion of free substrates by binding *only* at the active center of the enzyme. In certain cases, a depletion of the free substrates by nonspecific binding to the enzyme at sites other than the catalytic center may have to be considered. Although it seems reasonable to assume that the nonspecific binding is very weak (i.e., the dissociation constant of a nonspecific ES complex is very high compared to that of the "specific" ES complex), it may also be true that there are many sites for nonspecific binding, so that their effect may not be negligible. For example, there are about 120 nonspecific sites for pyruvate per molecule of pyruvic kinase (7). When the effect of such nonspecific binding is taken into consideration, extra terms must be added to Eq. 12 as presented in Appendix III. This effect may be difficult to evaluate experimentally; however, it should be considered as a possible source of error in a microdetermination of substrates, particularly when results obtained at low concentrations of enzymes are extrapolated to predict the rate at high concentrations of enzymes.

Another interesting theoretical possibility suggested by Eq. 13 is that one might be able to estimate the turnover number of an enzyme without being forced to use a homogeneous enzyme preparation. Once the turnover number is known, the molecular weight (or equivalent weight in the case of a multi-

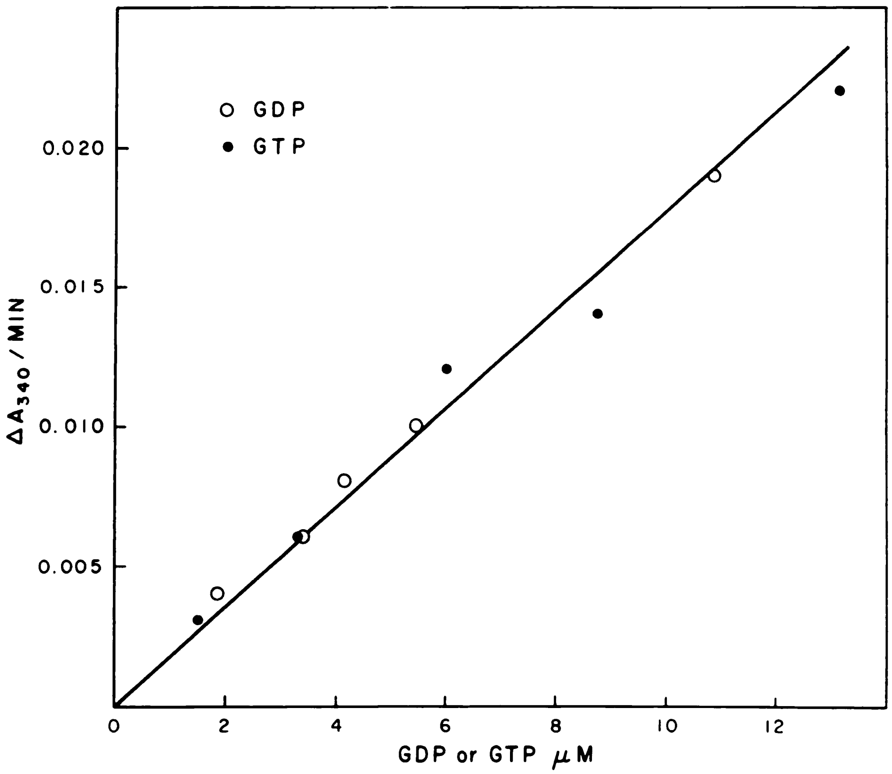


FIG. 4. *Rate of cycling in pyruvic kinase-succinic thiokinase system; plot of decrease in absorbance at 340 mμ versus the initial concentration of GDP or GTP*

The concentrations of the components in the reaction mixture were: succinate (Tris salt), pH 7.4, 50 mM; CoA, 0.1 mM; KCl, 100 mM; $MgCl_2$ 10 mM; phosphoenolpyruvate, 1.5 mM; DPNH, 0.1 mM; lactic dehydrogenase, 10 μg/ml; pyruvic kinase 8 μg/ml; succinic thiokinase, 2 μg/ml; and various amounts of either GDP or GTP. All components except the cycling substrates, guanine nucleotides, were mixed in a volume of 1 ml, and the reactions were started by the addition of either GDP (○) or GTP (●) solution in a volume less than 20 μl. The velocities are expressed as the decrease in absorbance at 340 mμ/min due to the oxidation of DPNH.

valent enzyme) can be calculated. The procedure is as follows: if one measures the reaction velocity with an increasing total amount of two enzymes, at a constant ratio of the two (α), e.g., $E_t = \alpha E_0$, $E'_t = (1 - \alpha)E_0$, then a plot of S_0/v *versus* $1/E_0$ will be a straight line, according to a reciprocal form of Eq. 13:

$$\frac{S_0}{v} = \left(\frac{1}{k_3} + \frac{1}{k'_3}\right) + \left(\frac{K}{k_3\alpha} + \frac{K'}{k'_3(1 - \alpha)}\right)\frac{1}{E_0}$$

When the line is extrapolated to the S_0/v axis, the intercept provides the harmonic mean of the turnover numbers of the two enzymes. Therefore, if one turnover number is known, the other can be calculated. In addition, if the molecular weight of the enzyme in question is known, one may employ this method to estimate the number of active sites per molecule.

APPENDIX I

An Approximation of the Solution for a Quadratic Equation Often Encountered in Enzyme Kinetics

Many fundamentally important equations in enzyme kinetics are quadratic, and their solutions include a square root term. In such cases, it may be too cumbersome to analyze the equations, therefore some important relationships deducible from the equations may be obscured. However, if these equations are expanded to power series, terms contributing the most become immediately obvious, and one can obtain an approximation of the desired degree of accuracy.

The general equation to be considered here is:

$$ax^2 - bx + c = 0 \qquad (1)$$

where a, b, and c are real numbers and equal to or greater than zero. The solution of Eq. 1 is given by:

$$x = \frac{b \pm \sqrt{b^2 - 4ac}}{2a} \qquad (2)$$

If the variable x represents a velocity or a concentration, it cannot be an imaginary number; therefore the discriminant, D, cannot be negative, i.e.

$$D = b^2 - 4ac \geq 0 \qquad (3)$$

The choice of sign in front of the square root term usually presents no problems. For example, if x can have a value of zero under a special condition, b and $\sqrt{b^2 - 4ac}$ must have opposite signs. Similar arguments had been used in the process of deriving Eq. 2 of Appendix II. Since the present concern is restricted to the case where b is positive,

$$x = \frac{b - \sqrt{b^2 - 4ac}}{2a} \qquad (4)$$

let $$z = ac/b^2 \qquad (5)$$

then, $$\sqrt{D} = \sqrt{b^2 - 4ac} = b(1 - 4z)^{1/2} \qquad (6)$$

A binomial expansion of Eq. 6 gives:

$$\sqrt{D} = b \sum_{m=0}^{\infty} \binom{1/2}{m} (1 - 4z)^m \qquad (7)$$

The condition for the convergence of this series, $|4z| \leq 1$, is satisfied by $D \geq 0$.

Equation 7 may be written as:

$$\sqrt{D} = b - 2bz\left(1 + \sum_{n=1}^{\infty} C_n z^n\right) \qquad (8)$$

where n represents the positive integer numbers, and

$$C_n = \frac{1 \cdot 3 \cdot 5 \cdot \cdot \cdot (2n - 1) \cdot 2^n}{(n + 1)!} = 1, 2, 5, 14, \text{etc.}$$

Substitutions of Eqs. 5 and 8 into Eq. 4 give:

$$x = \frac{c}{b}\left[1 + \sum_{n=1}^{\infty} C_n \left(\frac{ac}{b^2}\right)^n\right] \qquad (9)$$

or

$$x = \frac{c}{b}\left[1 + \frac{ac}{b^2} + \frac{2(ac)^2}{b^4} + \frac{5(ac)^3}{b^6} + \cdot \cdot \cdot\right] \qquad (10)$$

If ac/b^2 is small enough in comparison to 1, ac/b^2 and the other terms of higher powers can be neglected in Eq. 9, therefore:

$$x = c/b \qquad (11)$$

Notice that this first approximation of the solution of Eq. 1 is the same as the root of the linear equation, $-bx + c = 0$.

APPENDIX II

Generalized Rate Equation for Single-Enzyme Reaction

For the simple enzymic mechanism,

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \mathrm{P} \qquad (1)$$

Reiner (2) has derived the following rate equation by dropping one of the classic Michaelis-Menten assumptions; i.e., the amount of substrate which is bound by enzyme is very small compared with the total amount of substrate present in the mixture.

$$v = \frac{k_3}{2}\left[\,[(\mathrm{S}_t + \mathrm{E}_t + K) - \sqrt{(\mathrm{S}_t + \mathrm{E}_t + K)^2 - 4\,\mathrm{S}_t\mathrm{E}_t}\,\right] \qquad (2)$$

The hidden resemblance of this rate equation to the familiar Michaelis-Menten equation can be unveiled in the following manner. By a binomial expansion of the square root term, the above equation can be written as the following converging power series.

$$v = \frac{k_3\mathrm{S}_t\mathrm{E}_t}{\mathrm{S}_t + \mathrm{E}_t + K}\left(1 + \sum_{n=1}^{\infty} C_n z^n\right) \qquad (3)$$

where

$$C_n = \frac{1 \cdot 3 \cdot 5 \cdots (2n-1) \cdot 2^n}{(n+1)!} = 1, 2, 5, 14, \cdots$$

$$z = \mathrm{S}_t\mathrm{E}_t/(\mathrm{S}_t + \mathrm{E}_t + K)^2$$

When *either* S_t or E_t is much smaller than K, the terms included in the summation sign become negligible compared to 1, and the equation becomes

$$v = \frac{k_3\mathrm{S}_t\mathrm{E}_t}{\mathrm{S}_t + \mathrm{E}_t + K} \tag{4}$$

Finally, by restoring the omitted assumption, i.e., $\mathrm{E}_t \ll K$,

$$v = \frac{k_3\mathrm{S}_t\mathrm{E}_t}{\mathrm{S}_t + K} \tag{5}$$

which is the classic Michaelis-Menten equation.

And, if $\mathrm{S}_t \ll K$,

$$v = \frac{k_3\mathrm{S}_t\mathrm{E}_t}{\mathrm{E}_t + K} \tag{6}$$

which is analogous to the situation considered in the text. The same equation has been derived previously (3, page 35).

APPENDIX III

Effect of Depletion of Free Substrates by Nonspecific Binding on the Rate of a Cyclic Enzyme System

The depletion of free substrates by binding with the enzyme at sites other than the active center may significantly lower the velocity of an enzymic reaction under certain conditions. The theory of multiple equilibria between a substance and a protein has been extensively treated by Klotz (8). For the sake of simplicity, it will be assumed that there are n nonspecific binding sites on an enzyme-molecule, and each is uninfluenced by its neighbors, and furthermore, the dissociation constants are the same for each site. Under these conditions, the extent of combination of a substrate, S, with the enzyme at nonspecific sites may be expressed as:

$$r = \frac{n\mathrm{S}}{K_d + \mathrm{S}} \tag{1}$$

where r = moles of bound S/moles of total enzyme, S is the concentration of free substrate, and K_d is the dissociation constant of the nonspecific ES complex. If the nonspecifically bound substrate is designated by S_{ns}, and the total enzyme by E_t,

$$\mathrm{S}_{ns} = \frac{n\mathrm{E}_t\mathrm{S}}{K_d + \mathrm{S}} \tag{2}$$

If $\mathrm{S} \ll K_d$, which is the main concern of this paper, Eq. 2 may be reduced to:

$$\mathrm{S}_{ns} = n\mathrm{E}_t\mathrm{S}/K_d \tag{3}$$

When this relationship is applied to the model for the cyclic system under the conditions where Eq. 12 of the text is applicable, except the consideration of nonspecific bindings, the following equation is obtained:

$$v = \frac{1}{\dfrac{\mathrm{E}_t(1+\rho) + K}{k_3\mathrm{E}_t\mathrm{S}_0} + \dfrac{\mathrm{E}'_t(1+\rho') + K'}{k'_3\mathrm{E}'_t\mathrm{S}_0}} \tag{4}$$

and, if E has n_1 independent nonspecific binding sites for S with an identical dissociation constant of K_1, and n_2 sites for S′ with the dissociation constant of K_2, and similarly E′ has n_3 and n_4 binding sites with the respective dissociation constants of K_3 and K_4 for S and S′, then

$$\rho = \frac{n_1\mathrm{E}_t/K_1 + n_3\mathrm{E}'_t/K_3}{\mathrm{E}_t/\mathrm{K}}$$

$$\rho' = \frac{n_2\mathrm{E}_t/K_2 + n_4\mathrm{E}'_t/K_4}{\mathrm{E}'_t/\mathrm{K}'}$$

The similarity of Eq. 4 to Eq. 12 of the text is immediately obvious. It is interesting to note that ρ represents the ratio of the amount of S bound at nonspecific sites of both enzymes E and E′ to that amount bound at the active center of enzyme E; and ρ' is the ratio of S′ bound at nonspecific sites of both E and E′ to that amount bound at the active center of E′.

ACKNOWLEDGMENT

The authors wish to thank Professor R. E. Parks, Jr., for his encouragement and advice, and Mrs. Mária Décsy for her excellent technical assistance.

This work was supported by Grant No. T-94 G from the American Cancer Society.

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