Isotope Exchange Studies of the Mechanism of the Reaction Catalyzed by Adenosine Triphosphate: Creatine Phosphotransferase*

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SUMMARY

The mechanism of the reaction catalyzed by adenosine triphosphate: creatine phosphotransferase has been studied by measuring the initial velocities of the exchange with isotopically labeled substrates.

The rates of the creatine-phosphocreatine, ATP-ADP, and ADP-ATP exchanges at equilibrium are approximately equal and dependent on the concentrations of Mg$^{2+}$ and ADP$^{3-}$.

The ADP-ATP exchange rate increases hyperbolically to a maximum value as the concentration of the creatine-phosphocreatine pair is raised while the creatine-phosphocreatine exchange rate increases initially and then decreases with increasing concentrations of the MgATP-MgADP pair. The decrease in the exchange rate was shown to be due to the inhibitory effect of NaCl which is introduced when the reactants are formed from MgCl$_2$ and the sodium salts of the nucleotides.

The formation of a dead end enzyme-MgADP-creatine complex has been confirmed, but the experimental data were not in accord with the formation of a dead end enzyme-MgATP-phosphocreatine complex.

These data confirm the results from initial velocity and product inhibition studies. The results were consistent with the idea that the enzyme possesses two distinct sites, one for the nucleotide and the other for the guanidino substrate, and that the slowest step of the reaction in each direction is the interconversion of the central complexes, enzyme-MgATP-creatine and enzyme-MgADP-phosphocreatine. Thus, the reaction mechanism may be described as being rapid equilibrium, random (2). In addition, the kinetic data indicated that two “dead end” complexes, enzyme-MgATP-phosphocreatine and enzyme-MgADP-creatine, could be formed.

When using steady state kinetic investigations, it is difficult to be sure that the interconversion step is the rate-limiting one, although this conclusion can be reached as a first approximation. The isotope exchange technique of Boyer (3) and Boyer and Silverstein (4) is particularly useful for confirming this type of mechanism as it enables the rate of interconversion of the like substrate-product pairs to be determined. This technique has now been applied to the creatine kinase reaction, and it has been found that the initial rates of the ATP-ADP, creatine-phosphocreatine, and ADP-ATP exchanges at equilibrium are approximately equal and that they are influenced by the concentrations of Mg$^{2+}$ and ADP$^{3-}$ present at equilibrium (5). While the initial ADP-ATP exchange rate increased hyperbolically to a maximum value as the creatine-phosphocreatine pair was raised in concentration, higher concentrations of the MgATP-MgADP pair caused an inhibition of the exchange rate. This was shown to be due to the inhibitory effect of NaCl which is obtained when the Mg-nucleotide complexes are formed from MgCl$_2$ and the sodium salts of the nucleotides. The exchange pattern obtained by raising the concentration of the MgADP-creatine pair was consistent with the formation of a dead end enzyme-MgADP-creatine complex, but it appears unlikely that a second dead end complex, enzyme-MgATP-phosphocreatine, is formed.

The mechanism of the reaction catalyzed by adenosine triphosphate: creatine phosphotransferase (EC 2.7.3.2; creatine kinase),

\[
\text{MgADP}^- + \text{phosphocreatine} \rightleftharpoons \text{MgATP}^{2-} + \text{creatine}
\]

has been investigated by Morrison and James (1) with initial velocity and product inhibition studies. The results were consistent with the idea that the enzyme possesses two distinct sites, one for the nucleotide and the other for the guanidino substrate, and that the slowest step of the reaction in each direction is the interconversion of the central complexes, enzyme-MgATP-creatine and enzyme-MgADP-phosphocreatine. Thus, the reaction mechanism may be described as being rapid equilibrium, random (2). In addition, the kinetic data indicated that two “dead end” complexes, enzyme-MgATP-phosphocreatine and enzyme-MgADP-creatine, could be formed.

When using steady state kinetic investigations, it is difficult to be sure that the interconversion step is the rate-limiting one, although this conclusion can be reached as a first approximation. The isotope exchange technique of Boyer (3) and Boyer and Silverstein (4) is particularly useful for confirming this type of mechanism as it enables the rate of interconversion of the like substrate-product pairs to be determined. This technique has now been applied to the creatine kinase reaction, and it has been found that the initial rates of the ATP-ADP, creatine-phosphocreatine, and ADP-ATP exchanges at equilibrium are approximately equal and that they are influenced by the concentrations of Mg$^{2+}$ and ADP$^{3-}$ present at equilibrium (5). While the initial ADP-ATP exchange rate increased hyperbolically to a maximum value as the creatine-phosphocreatine pair was raised in concentration, higher concentrations of the MgATP-MgADP pair caused an inhibition of the exchange rate. This was shown to be due to the inhibitory effect of NaCl which is obtained when the Mg-nucleotide complexes are formed from MgCl$_2$ and the sodium salts of the nucleotides. The exchange pattern obtained by raising the concentration of the MgADP-creatine pair was consistent with the formation of a dead end enzyme-MgADP-creatine complex, but it appears unlikely that a second dead end complex, enzyme-MgATP-phosphocreatine, is formed.

The basic mechanism of the reaction catalyzed by creatine kinase at pH 8.0 can be described as being rapid equilibrium, random (1) and illustrated by the shorthand method of Cleland (2) as follows

\[
\text{MgADP}^- + \text{phosphocreatine} \rightleftharpoons \text{MgATP}^{2-} + \text{creatine}
\]
The kinetics of isotope exchange are thus similar to those obtained in the exchange reaction. In the presence of high concentrations of the nucleotide substrates, the enzyme would be forced into the forms, enzyme-MgATP and enzyme-MgADP, and the flux would occur by the upper pathway of Scheme 1. Since labeled enzyme reacts with enzyme MgATP, the exchange velocity would increase to a maximum as the concentration of this form of enzyme rises to a maximum value. The situation would be similar for the ADP-ATP (or ATP-ADP) exchange as the concentration of the creatine-phosphocreatine pair was increased and reaction would occur via the lower pathway of Scheme 1. If the interconversion of enzyme-MgADP-phosphocreatine to enzyme-MgATP-creatine is the slowest step of Scheme 1 from left to right, then under all of the conditions, near or far from equilibrium, the initial velocity of the phosphocreatine-creatine exchange must equal that of the ADP-ATP exchange. Similarly, if the conversion of enzyme-MgATP-creatine to enzyme-MgADP-phosphocreatine is the slowest step of Scheme 1 from right to left, the initial exchange velocities for creatine-phosphocreatine and ATP-ADP must be equal. When the system is at equilibrium, the initial rates of the ADP-ATP and ATP-ADP exchanges must be equal, as must the initial rates of the creatine-phosphocreatine and phosphocreatine-creatine exchanges. Hence, under these conditions, all of the exchanges must be equal and this is a characteristic feature of a rapid equilibrium, random mechanism.

When the concentrations of the pairs of reactants involved in the formation of dead end complexes are raised, a different pattern is obtained. Initially the exchange velocity will increase as the concentration of the enzyme species with which the labeled compound reacts increases in concentration. But at higher concentrations of this pair of reactants, the enzyme is forced into an inactive dead end complex which results in an eventual decrease of the exchange velocity to zero when saturation is reached.

**Derivations of Initial Velocity Equations for Isotope Exchange**

The Bi Bi rapid equilibrium, random mechanism with two dead end complexes (2), as expressed by Reactions I to III, may be considered in general terms taking $A$, $B$, $P$, and $Q$ to represent MgADP, phosphocreatine, creatine, and MgATP, respectively. The initial velocity of the exchange reactions, $A^* \rightarrow Q^*$ or $B^* \rightarrow P^*$ (where asterisk (*) represents labeled reactant) may be written as

$$v = k_1 (EAB^*)$$

irrespective of whether or not the system is at equilibrium. The concentration of $EAB$ may be expressed in terms of the concentrations of the various enzyme forms and total enzyme ($e_t$) so that
where $V_1$, which is equal to $k_{+1}$, represents the maximum velocity of the chemical reaction in the forward direction as formulated in Scheme 1, and the kinetic constants are as defined for Reactions 1 to III (1).\(^1\)

Under equilibrium conditions, the Haldane relationship

$$K_{eq} = \frac{V_1 K_p K_q}{V_2 K_a K_b}$$

(where $V_2$, which is equal to $k_{+2}$, represents the maximum velocity of the reverse chemical reaction as formulated in Scheme 1) and the steady-state relationships, $K_1 K_8 = k_{+7} K_{ib}, K_{ib} K_q = K_p K_{ib}, K_{ib} K_p = K_q K_{ib}$, and $K_{ib} K_q = K_q K_{ib}$ (1) may be used to simplify the equation to

$$v = \frac{K_8}{1 + \frac{K_8}{B} + \frac{K_9}{AB} + \frac{K_{10} K_{11}}{K_8 K_{12} B} + \frac{K_{13} K_{14} P}{K_{15} K_{16} B} + \frac{K_{17} K_{18} P Q}{K_{19} K_{20} B} + \frac{K_{21} K_{22} Q}{K_{23} K_{24} B} + \frac{K_{25} K_{26} Q}{K_{27} K_{28} B} + \frac{K_{29} K_{30} Q}{K_{31} K_{32} B} + \frac{K_{33} K_{34} Q}{K_{35} K_{36} B}}$$

$$V_1$$

(1)

$$v = \frac{K_8}{1 + \frac{K_8}{B} + \frac{K_9}{A} + \frac{K_{10} K_{11}}{K_8 K_{12} B} + \frac{K_{13} K_{14} P}{K_{15} K_{16} B} + \frac{K_{17} K_{18} P Q}{K_{19} K_{20} B} + \frac{K_{21} K_{22} Q}{K_{23} K_{24} B} + \frac{K_{25} K_{26} Q}{K_{27} K_{28} B} + \frac{K_{29} K_{30} Q}{K_{31} K_{32} B} + \frac{K_{33} K_{34} Q}{K_{35} K_{36} B}}$$

(2)

An identical expression is obtained under equilibrium conditions for the initial velocity of the $Q^* \rightarrow A^*$ and $P^* \rightarrow B^*$ exchanges and thus Equation 2 describes the initial velocity, under equilibrium conditions, of all of the exchanges for a rapid equilibrium reaction with two dead end complexes.

The hyperbolic increase in exchange velocity as like substrate-product pairs are raised in concentration can be illustrated by considering the velocity of the $B-P$ exchange as a function of the concentration of $A$ while maintaining the $A$ to $Q$ ratio constant. $Q$ can be eliminated from Equation 2 by means of the Haldane relationship, and rearrangement of the resulting equation in reciprocal form with $A$ as the variable substrate gives

$$\frac{1}{v} = \frac{K_8}{V_1} \left[ 1 + \frac{K_8}{B} \left( 1 + \frac{P}{K_{15}} \right) \right] + \frac{1}{V_1} \left[ 1 + \frac{K_9}{A} \right]$$

which is the equation of a straight line when $1/v$ is plotted against $1/A$.

It should be noted that the formation of the dead end complexes, EAP and EBQ, represented in Equation 2 by $(V_1 K_{18})/K_{19} K_{20}$ and $(V_2 K_{18})/K_{21} K_{22}$, respectively, does not affect the hyperbolic increase of the exchange velocity when like substrate product pairs $(A-Q, B-P)$ are raised in concentration as the ratios of $A$ to $Q$ and $B$ to $P$ remain constant. However, when the unlike substrate-product pairs associated with the formation of dead end complexes $(A-Q, B-P)$ are raised in concentration, $A$ or $B$ tends to infinity with the result that the velocity will tend to zero.

The above exchange velocity equations apply only under conditions similar to those used by Morrison and James (1) where the concentrations of Mg\(^{2+}\), Cl\(^-\), and ADP\(^-\) were sufficiently low as not to give rise to inhibition. However, this was not true for all of the conditions under which equilibrium exchange rates were measured in the present work and hence a general velocity equation to describe the exchange rate must take into account the formation of dead end enzyme complexes which arise as a result of the following.

The reaction of ADP\(^-\) (Scheme 1) with the free form of enzyme as well as with the enzyme-phosphocreatine and enzyme-creatine complexes: It was considered that all of the complexes involving enzyme and inhibitor had the same dissociation constant ($K_2$) which was taken to be 0.25 mM (5). The reaction of MgCl\(_2\) (M) at a site, other than the active site, on the free enzyme, enzyme-MgADP, enzyme-MgADP-creatine, and enzyme-ADP ($K_3$); enzyme-phosphocreatine, enzyme-MgADP-phosphocreatine, and enzyme-ADP-phosphocreatine ($K_4$); enzyme-creatine and enzyme-ADP-creatine ($K_5$); enzyme-MgATP-creatine, enzyme-MgATP, and enzyme-MgATP-phosphocreatine ($K_6$): The dissociation constants associated with each enzyme complex are indicated in brackets and the values used for $K_4$ and $K_5$ were 10.5 mM and 21 mM, respectively, as reported by Morrison and O’Sullivan (5). Values of 14 mM and 31 mM were taken for $K_4$ and $K_5$, respectively.\(^2\)

The reaction of NaCl (Cl) with enzyme-MgADP, enzyme-ADP, Mg-enzyme-MgADP, and Mg-enzyme-ADP ($K_7$); enzyme-phosphocreatine and Mg-enzyme-phosphocreatine ($K_8$); enzyme-creatine and Mg-enzyme-creatine ($K_9$); enzyme-MgATP and Mg-enzyme-MgATP ($K_{10}$): The values used for the dissociation constants which are indicated in brackets were: $K_5$, 50 mM, $K_6$, 100 mM, $K_7$, 70 mM, and $K_8$, 80 mM.\(^3\)

When allowance is made for the formation of the above enzyme complexes, the velocity equation for all of the exchanges at equilibrium can be seen at the top of the following page. This equation was used for the calculation of the exchange velocities to be expected under various equilibrium conditions (cf. Table I), but in view of its complexity no attempt was made to compare the theoretical and experimental values for the velocity of Cl with all of the ternary enzyme complexes has been considered to be negligible. It has been assumed that Mg and Cl react independently with the binary enzyme forms since no information was available on this point. Further, it has been assumed that Cl reacts to the same extent with enzyme-ADP as with enzyme-MgADP.

\(^1\) J. F. Morrison and E. James, unpublished results.

\(^2\) Taken from the unpublished work of Morrison and James which also showed that there was only a slight reaction of Cl with free enzyme and the ternary enzyme complexes, enzyme-MgADP-phosphocreatine and enzyme-MgATP-creatine. Hence, the reaction of Cl with all of the ternary enzyme complexes has been considered to be negligible. It has been assumed that Mg and Cl react independently with the binary enzyme forms since no information was available on this point. Further, it has been assumed that Cl reacts to the same extent with enzyme-ADP as with enzyme-MgADP.
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FIG. 1. Determination of the maximum velocity of the creatine kinase reaction by simultaneously varying the concentration of MgADP- and phosphocreatine while maintaining their ratio constant. Reaction mixtures (1.0 ml) contained 0.1 M triethanolamine-HCl buffer (pH 8.0), 0.01 mM EDTA, and 0.5 μg of creatine kinase in addition to the indicated concentrations of MgADP- and phosphocreatine. The concentration of free Mg2+ was maintained constant at 1.0 mM; temperature, 30°. Velocity is expressed as micromoles of creatine released per μg of enzyme per min.

EXPERIMENTAL PROCEDURE

Materials

Chemicals—The sodium salts of ADP and ATP, obtained from P-L Biochemicals, showed the presence of only single spots which absorbed ultraviolet light after chromatography in isobutyric acid-NH3 (sp. gr. 0.88)-water (66:1:33, v/v) and were used without further purification. Stock solutions of both nucleotides (80 μM) were adjusted to pH 7.6 with 1 N NaOH and stored at −10°. Their concentrations were checked by measurement of the absorption at 259 μm in 0.01 N HCl (6). Phosphocreatine (Calbiochem), which contained 0.5% (mole per mole) of free creatine, was used without further purification for the exchange studies, but it was recrystallized as described by Morrison, O'Sullivan, and Ogston (7) for the determination of enzyme activity. Creatine was purchased from Calbiochem, triethanolamine from Eastman Organic Chemicals, and formic acid from Mallinckrodt Chemical Works. MgCl2 and EDTA were certified reagents obtained from Fisher Scientific Company. Solutions of MgCl2 were standardized as previously described (7), and solutions of EDTA were adjusted to pH 8.0 with 1 N NaOH.

8-14C-ATP (8.9 or 10.0 mCi per mmole) and 8-14C-ADP (34 mCi per mmole) were purchased from Schwarz BioResearch, Inc. and 1-14C-creatine was obtained from Calbiochem.

Creatine Kinase—This was a crystalline preparation prepared in Canberra, Australia by the method of Kuby, Noda, and Lardy (8). The crystals were dissolved in 0.01 M glycine buffer (pH 9.0), dialyzed against the same buffer, and freeze-dried before being air-mailed to Madison, Wisconsin. The dried material (30 mg) was cooled in a Dry Ice-ethanol mixture, and 1.0 ml of 0.001 M triethanolamine-HCl buffer containing 1 mM EDTA (pH 8.0) was added to it. The solid mixture was triturated until the temperature reached 2°, whereupon the insoluble material was removed by centrifuging. The stock enzyme solution, containing 24.2 mg of protein per ml, was stored at 2°. Enzyme activity was determined as described below.

Methods

Determination of Protein—Protein concentrations were determined by the method of Gornall, Bardawill, and David (9) with crystalline bovine serum albumin as the standard.

Measurement of Creatine Kinase Activity—In order to calculate the expected rate of isotope exchange, it was necessary to know the maximum velocity of the chemical reaction in one direction as obtained with the redissolved, freeze-dried preparation referred to in “Materials.” This was determined for the reaction in the forward direction by measuring the rate of release of creatine from phosphocreatine (7) under conditions where the concentrations of MgADP- and phosphocreatine were varied simultaneously while maintaining the ratio of the two substrates constant. As previously reported (1), the initial velocity of the forward reaction can be written in reciprocal form as

\[
\frac{1}{v} = \frac{1}{V_1} \left( \frac{K_{m_a} K_5}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 \right)
\]

where \(K_{m_a}\) is the dissociation constant for enzyme-MgADP, \(K_a\) and \(K_b\) are the Michaelis constants for MgADP- (A) and phosphocreatine (B), respectively, and \(V_1\) is the maximum velocity. When \(B\) is allowed to equal \(A\), the equation becomes

\[
\frac{1}{v} = \frac{K_{m_a} K_5}{V_1} \left( \frac{1}{B} \right)^3 + \frac{K_a}{V_1} + \frac{K_b}{V_1} + \frac{1}{V_1}
\]

so that a plot of 1/v against 1/B gives a parabola. The data of Fig. 1 were fitted to a parabola with the computer program of Cleland (10) and the maximum velocity for this enzyme preparation was found to be 0.107 pmole of creatine per μg of enzyme per min. This may be compared with a value of 0.208 pmole...
per μg of enzyme per min, as reported by Morrison and James (1) for a preparation which had not been subjected to freeze-drying.

**Determination of Exchange Rates at Equilibrium**—All of the experiments were carried out at pH 8.0 and 30° in the presence of 0.1 M triethanolamine-HCl and 0.01 mM EDTA with the enzyme usually at a concentration of 2 μg per ml. The total concentrations of Mg, ATP, ADP, creatine, and phosphocreatine required to give equilibrium concentrations of the various reactant species were taken from Table III of the Addendum of the article by Kuby and Noltmann (11).

Trial experiments to determine the reaction times and enzyme concentration were not necessary. An estimate of the initial exchange rate to be expected under the various equilibrium conditions could be calculated by substituting the equilibrium concentrations of the reactants (11) and the values of the kinetic constants (1) into the simplified velocity Equation 2. Although the maximum velocity of the reaction in the forward direction with the freeze-dried enzyme was less than that obtained previously, the ratio of the maximum velocities of the reaction in each direction ($V_1/V_2$) should not change. The value of this ratio was taken to be 1.888 (1). Since the equilibrium concentrations of the reactants were known (11), the equilibrium distribution of the counts between substrate and product could be calculated. Then by assuming that the exchange reaction would be linear for 30% of the way to equilibrium, the time over which the reaction might be expected to be linear was determined. Samples were taken at appropriate intervals.

For studies on the exchange velocity of raising the concentration of one pair of reactants, it was sometimes necessary to modify the equilibrium concentrations of the reactants (11), since calculation showed that under these conditions the expected rate of exchange was close to the maximum value. Each reactant of the pair was reduced by the same factor so as to maintain the equilibrium, and at the same time, free Mg2+ was held constant at the original concentration. When nucleotides were involved, the concentrations of the Mg-nucleotide complex and the free nucleotide were reduced to the same extent so as to keep their ratio constant and the total Mg added was reduced by an amount equal to the difference between the original and new concentration of Mg-nucleotide. The concentration of free phosphocreatine was reduced in a similar manner, but as creatine does not form complexes with Mg2+, the reduction of its concentration did not necessitate any correction of the Mg added.

The basic reaction components at the required concentrations were mixed in a stock solution from which equal samples were pipetted into a series of test tubes. Additional amounts of the pair of reactants being raised in concentration were then added so that the final volume was either 0.48 or 0.96 ml. To raise the concentration of MgATP, equal amounts of both Mg and ATP were added. It was considered that the apparent stability constant for MgATP− of 70,000 M−1 (11, 12) was sufficiently high to allow the concentration of ATPr− to be neglected. In addition, ATPr− has little effect on the reaction. However, when raising the concentration of MgADP, it was necessary to take into account the ADP− concentration as the apparent stability constant for MgADP− has a relatively low value of 3,000 to 4,000 M−1 (11, 12). Thus, the concentrations of MgADP− and ADP− were increased by the same factor and the total ADP (S) added represented the sum of the concentrations of the two species. The additional amount of MgCl2 (M) to be added so as to give the required concentration of MgADP while maintaining a constant concentration of Mg2+ (μ) was determined from the relationship

$$M - \frac{μS}{K + μ}$$

where K represents the dissociation constant of the MgADP− complex. The value used was 0.33 mM (11). Similar calculations were made when the concentration of free phosphocreatine was raised, although because of the relatively low apparent stability constant for Mg-phosphocreatine of 40 M−1 (11, 12), the corrections were negligible in most instances.

The equilibrium mixture of the reactants was incubated for 3 min at 30° after which either 0.02 or 0.04 ml of enzyme, previously diluted in cold 1 mM triethanolamine-HCl buffer containing 0.01 mM EDTA (pH 8.0), was added. After a further 10 min, the exchange reaction was started by the addition of 10 to 40 μl of labeled substrate from a microsyringe (Hamilton Company, Inc., Whittier, California). Samples of the reaction mixture were taken at appropriate intervals with either a 50- or 100-μl syringe of the same type and rapidly applied to DEAE-cellulose paper, as described below, so as to stop the reaction. A finite time was required for the sample to diffuse into the paper and this was related to the volume of the sample. However, as at least five samples of the same volume were taken and the initial velocity of the exchange was determined from the slope of the line for a plot of counts per min against time, it was not necessary to take into account the constant time increment. Application of the samples to DEAE-cellulose paper was found to stop the reaction, and presumably, this is attributable to the absorption of the negatively charged reactant onto the DEAE groups.

**Separation and Counting of ADP and ATP**—The reaction was stopped by application of samples (10 to 40 μl containing up to 160 mmoles of each nucleotide) of the reaction mixture to sheets (7 × 7 in) of Whatman DEAE-cellulose paper to which four strips (1 in wide) of Whatman No. 3MM paper were stapled at the top. After drying in air, the paper was folded into a cylinder around a support which fitted into the lid of a Kerr Mason jar. The chromatogram was placed inside the Mason jar and developed by ascending chromatography in 0.6 M ammonium formate containing 5 mM EDTA (pH 3.1) for 2½ hours at 23°. After drying in air overnight, the chromatogram was heated to 10 min at 60°. The location of the nucleotide spots was detected with the aid of ultraviolet light; after marking, strips (2 × 1 in) containing the nucleotides were cut out. These were fluffed lengthwise, immersed in 15 ml of scintillator (3 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5’-phenyloxazolyl) benzene in 1 liter of toluene) and counted in a Packard Tri-Carb liquid scintillation counter with an over-all efficiency of 38%. The geometry of the paper had no effect on the counting efficiency and the counts were proportional to the sample of 14C-nucleotide added. When necessary, additional amounts of nucleotide marker were added to the chromatogram before development to facilitate location of the spot.

The specific radioactivities of ATP and ADP were determined by adding the counts for the ADP and ATP spots and dividing by the total concentration of ATP or ADP present in the reaction.

We are grateful to Dr. R. M. Bock for helpful suggestions with regard to the chromatography of nucleotides on DEAE-cellulose paper.
The specific radioactivity of creatine was determined from the total creatine present in the reaction mixture and the number of counts obtained when samples of the reaction mixture were applied to disks and counted without prior washing.

Calculation of Initial Exchange Velocities—These were determined from the following relationship

\[
\frac{\text{counts per min of product per min per } \mu\text{g of enzyme}}{\text{specific radioactivity of substrate}} = \text{velocity (micromoles per min per } \mu\text{g of enzyme)}
\]

Analysis of Data—The type of curve obtained was determined by graphical plots of the data which were then analyzed by the appropriate computer program of Cleland (10), with an IBM 1620 computer. The lines in the figures were drawn by using the constants so obtained. A computer program was also used to calculate from the total concentrations of the reactants, the concentrations of the various ionic species which would be present in an equilibrium mixture when it was assumed that the only metal complexes formed were MgATP\textsuperscript{2-}, MgADP\textsuperscript{-}, and Mg-phosphocreatine. For this purpose, stability constants of 70,000 and 9000 m\textsuperscript{-1}, 4000 m\textsuperscript{-1}, and 40 m\textsuperscript{-1} were used for MgATP\textsuperscript{2-}, MgADP\textsuperscript{-}, and Mg-phosphocreatine, respectively. Unless otherwise stated, the values so obtained were used for the theoretical calculations.

RESULTS

Linearity of Exchange Velocities with Time and Enzyme Concentration—The rates of the ATP-ADP, ADP-ATP, and creatine-phosphocreatine exchanges were linear with time (Fig. 2) even under conditions where the blank values were high relative to the slope of the line. The initial exchange rate was proportional to the enzyme concentration as determined by measurement of the creatine-phosphocreatine exchange (Fig. 3).

Exchange Rates under Various Equilibrium Conditions—The initial velocities of the ATP-ADP, ADP-ATP, and creatine-phosphocreatine exchanges were determined under three sets of equilibrium conditions (Table I), and it will be noted that, under any particular set of conditions, the three exchange rates are approximately equal as required for a rapid equilibrium, random mechanism. Furthermore, the rates are similar to those calculated by substitution of the equilibrium concentrations of the various ionic species, which function as substrates and the kinetic constants associated with these species, into Equation 4. Two sets of values for the equilibrium concentrations of the substrates were used; those reported by Kuby and Noltmann (11) and those calculated on the basis that the only metal complexes present in the reaction mixture were MgATP\textsuperscript{2-}, MgADP\textsuperscript{-}, and Mg-phosphocreatine. It is interesting that the exchange velocities for the two calculations are similar.

Although the exchange rates are approximately equal under a
TABLE I  
Equilibrium exchange rates for creatine kinase reaction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of Mg(^{2+}) (mM)</th>
<th>Concentration of ADP(^{2-}) (mM)</th>
<th>Calculated exchange rate (mumoles/min/(\mu)g)</th>
<th>Determined exchange rate (mumoles/min/(\mu)g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.04</td>
<td>0.02</td>
<td>1.09</td>
<td>1.64</td>
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<tr>
<td>II</td>
<td>1.01</td>
<td>1.27</td>
<td>0.24</td>
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<td>III</td>
<td>0.9</td>
<td>14.8</td>
<td>0.03</td>
<td>0.02</td>
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</tbody>
</table>

* Weighted mean of four determinations.

Fig. 4. a, effect of increasing the concentration of the creatine-phosphocreatine pair on the initial velocity of the ADP-ATP exchange. b, double reciprocal plot of the data given in a. The basic reaction mixture contained in 0.5 ml, 0.1 M triethanolamine-HCl buffer (pH 8.0), 3.85 mM ATP, 1.09 mM ADP, 6.0 mM MgCl\(_2\), 2.09 mM creatine, 0.335 mM phosphocreatine, 0.01 mM EDTA, and 1 \(\mu\)g of creatine kinase. The concentrations of creatine and phosphocreatine were increased as indicated in the figure. The exchange reaction was started by the addition of 20 \(\mu\)l of \(^{14}\)C-ADP (0.20 \(\mu\)C; 0.006 \(\mu\)mole), or 20 \(\mu\)l of \(^{14}\)C-ATP (0.20 \(\mu\)C; 0.02 \(\mu\)mole). The expected exchange rates were determined with the concentrations of the various ionic species present in solution which were taken from (a) the data of Kuby and Noltmann (11) and (b) calculations which assumed that the only metal complexes present in solution were MgATP\(^{2-}\), MgADP\(^{2-}\), and Mg-phosphocreatine. The values of the kinetic constants for the substrates were taken from Morrison and James (1) while inhibitor constant values were those given under "Methods."

Product Pairs—A rapid equilibrium, random mechanism for a two substrate-two product reaction is also characterized by the fact that the exchange rate for one substrate-product pair will increase hyperbolically as the other pair is raised in concentration. Fig. 4a shows that the ADP-ATP exchange rises hyperbolically to a maximum value as the concentration of the creatine-phosphocreatine pair is increased. The hyperbolic nature of the plot is confirmed by the finding that when the data of Fig. 4a is plotted in reciprocal form (Fig. 4b) a straight line is obtained. It was not possible to use higher concentrations of the creatine-phosphocreatine pair because of the limited solubility of creatine.

A similar picture was obtained for the creatine-phosphocreatine
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Formation of Dead End Complexes—The formation of a dead end complex involving enzyme-MgADP-creatine was shown by increasing the concentration of the MgADP-creatine pair and determining the rate of the ATP-ADP exchange. Fig. 7a shows that the exchange rate rises to a maximum value and then decreases as more and more enzyme is tied up in the form of a dead exchange by raising the concentration of the MgATP-MgADP
pair, up to a certain concentration of the nucleotides (Fig. 5a). That is, reciprocal plots with two different equilibrium concentrations of creatine gave straight lines which cut the abscissa at approximately the same point to give complex constant values of 0.17 and 0.14 nM. However, at higher concentrations of the nucleotide pair, there developed an inhibition of the exchange which is not in accord with a rapid equilibrium, random reaction. This finding prompted an investigation of the effect of higher concentrations of MgADP and MgATP on the initial velocity of the chemical reaction in the forward and reverse directions, respectively. Concentrations of MgADP up to 6.0 mM, which corresponded to the highest concentration of the compound used in the exchange experiments, did not cause any inhibition (Fig. 6c). On the other hand, high concentrations of MgATP, similar to those used to obtain the results of Fig. 5a, did cause inhibition (Fig. 6b). This inhibition was not due to the substrate itself since it could be accounted for by the presence of NaCl which is introduced when MgATP is formed from MgCl2 and the sodium salt of ATP. That is, the experimental points could be fitted to the theoretical velocity curve obtained by allowing for the inhibition of the reaction by NaCl (Fig. 6b). The experimental exchange data of Fig. 5a also gave a reasonable fit to the theoretical curve obtained by using Equation 4 which allows for the inhibitory effect of NaCl (Fig. 5b). Thus, the exchange results are not inconsistent with the creatine kinase reaction having a rapid equilibrium, random mechanism and at the same time, they point to the need of taking into consideration the possibility of such inhibitory effects when drawing conclusions from isotope exchange studies.

Fig. 5. a, double reciprocal plots of the effect of raising the concentrations of the MgATP-MgADP pair on the initial velocity of the creatine-phosphocreatine exchange. b, comparison of the experimental data of a with the theoretical curve obtained by calculation with Equation 4. The theoretical values for 1/v were multiplied by 1.19. Reaction mixtures contained 1.0 ml, 0.1 M triethanolamine-HCl buffer (pH 8.0), 0.01 mM EDTA, 0.097 mM ADP, 0.362 mM ATP, 2.13 mM MgCln, and either 4.18 mM creatine and 0.756 mM phosphocreatine (O—O) or 2.09 mM creatine and 0.378 mM phosphocreatine (O—O). The concentrations of MgATP2- and MgADP- were increased as indicated in the figure. The exchange reaction was started by the addition of 10 μl of 14C-creatine (0.5 μC). Creatine kinase, 2 μg; temperature, 30°. Exchange rate (V) is expressed as millimicromoles per min per μg of enzyme.

Fig. 6. Effect of increasing concentrations of MgADP- (a) and MgATP+ (b) on the initial velocity of the chemical reactions in the forward and reverse directions, respectively. Reaction mixtures contained 0.1 M triethanolamine-HCl buffer (pH 8.0), 0.01 mM EDTA, and either (a) 0.41 μg of enzyme, 10 mM phosphocreatine, and the indicated concentrations of MgADP- or (b) 1.16 μg of enzyme, 16 mM creatine, and the indicated concentrations of MgATP+. The enzyme preparation had not been freeze-dried. The concentration of free Mg2+ was maintained constant at 1.0 mM. Volume, 1.0 ml; temperature, 30°. Velocity is expressed as micromoles per min per μg of enzyme.
FIG. 7. a, effect of increasing concentrations of the MgADP-creatine pair on the initial velocity of the ATP-ADP exchange. b, comparison of the experimental data of a, plotted in reciprocal form with the theoretical curve obtained with Equation 4. The theoretical values for 1/v were multiplied by 1.55. Basic reaction mixtures contained in 0.5 ml: 0.1 m triethanolamine-HCl buffer (pH 8.0), 0.01 mm EDTA, 3.85 mm ATP, 0.323 mM ADP, 5.52 mM MgCl₂, 1.39 mM creatine, 0.756 mM phosphocreatine, and 1 µg of creatine kinase. The concentrations of MgADP and creatine were increased as indicated in the figure. The exchange reaction was started by the addition of 40 µl of ¹⁴C-ATP (0.4 µC); temperature, 30°. Exchange rate (V) is expressed as millimicromoles per min per µg of enzyme.

end complex which is not available for reaction with ¹⁴C-ATP. When the data of Fig. 7a are plotted in reciprocal form (Fig. 7b), a nonrectangular hyperbola, which corresponds to the general equation for substrate inhibition, is obtained. The general features of this plot correspond well with those of a theoretical curve obtained by using Equation 4.

It should be noted that the amount of NaCl introduced at the highest concentration of MgADP was such as to cause only negligible inhibition of the exchange reaction.

Similar experiments to that elaborated above were carried out to detect the formation of a dead end enzyme-MgATP-phosphocreatine complex. When the MgATP-phosphocreatine pair was raised in concentration, both the creatine-phosphocreatine and ADP-ATP exchange rates rose and then fell (Fig. 8a). However, the rates of the two exchanges, which are associated with the forward and reverse reactions, were not equal. This suggests that the system was not at equilibrium, but the interpretation of the results would not be affected for, as previously indicated (see "Theory"), it is not essential in such studies that the system by precisely at equilibrium.

Although the results of Fig. 8a can be interpreted as indicating the formation of a dead end enzyme-MgATP-phosphocreatine complex, it is necessary to take into account the fact that the inhibition was obtained at relatively high concentrations of MgATP. Thus, the inhibition could be independent of the phosphocreatine concentration and due to the introduction of NaCl. To delineate this point, a comparison was made between the experimental data for the creatine-phosphocreatine exchange and the theoretical curves obtained with Equation 4 when $K_{1b}$ was set equal to 18 mM (1) and infinity. The results illustrated in Fig. 8b suggest that the inhibition can be accounted for by the inhibitory effect of NaCl alone. Therefore, the formation of an enzyme complex involving the two phosphoryl group donors of the reaction appears unlikely.

**DISCUSSION**

The results of the investigation of the reaction catalyzed by creatine kinase by means of isotope exchanges are in accord with the previous conclusions of Morrison and James (1) that the reaction catalyzed by this enzyme has a rapid equilibrium, random mechanism. They are also in agreement with the conclusion that a dead end enzyme-MgADP-creatine complex can be formed, but there was no evidence which could be taken as being indicative of the formation of a second dead end complex involving enzyme, MgATP, and phosphocreatine. In this connection, it is interesting to note that the recent isotope exchange studies of Fromm, Silverstein, and Boyer (14) have confirmed the previous conclusion of Fromm and Zewe (15) that hexokinase catalyzes a rapid equilibrium, random reaction. It is also of interest that the kinetic data of Fromm and Zewe (15) suggest that a dead end enzyme-MgADP-glucose complex can be formed.

The agreement of the results obtained by product inhibition and isotope exchange studies of the creatine kinase reaction points to the value of the latter technique. This is especially true for studying enzymes which are believed to have a rapid equilibrium, random mechanism since it is possible to obtain a direct measure of the rate of interconversion of the central complexes which would be the slowest step of the reaction. The complexity of studies of phosphotransferase reactions has also become apparent. As previously pointed out (1), kinetic investigations of phosphotransferase reactions are complicated by the fact that the essential divalent metal ion undergoes non-enzymic reactions with the substrates and because particular ionic species, such as Mg²⁺ and ADP⁻⁷, can be inhibitory. But with initial velocity studies in the absence and presence of product
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FIG. 8. a, double reciprocal plot of the effect of increasing concentrations of the MgATP-phosphocreatine pair on the initial velocities of the ADP-ATP (O—O) and creatine-phosphocreatinine (●—●) exchanges. b, comparison of the experimental data for the creatine-phosphocreatine exchange (a) with the theoretical curves obtained with Equation 4 when \( K_{n} \) was set equal to 18 mM (upper curve) and infinity (lower curve). The basic reaction mixture contained: 0.1 M triethanolamine-HCl buffer (pH 8.0), 0.01 mM EDTA, 1.085 mM ATP, 0.097 mM ADP, 2.85 mM MgCl\(_{2} \), 2.09 mM creatine, and 1.00 mM phosphocreatine. The concentrations of MgATP\(^{2-} \) and phosphocreatine were increased as indicated in the figure. The creatine-phosphocreatine exchange reaction was started by the addition of 10 \( \mu \)l of \(^{14} \)C-creatine (0.5 \( \mu \)Ci) to 1.0 ml of reaction mixture containing 2 \( \mu \)g of creatine kinase. The ADP-ATP exchange reaction was started by the addition of 15 \( \mu \)l of \(^{14} \)C ADP to 0.5 ml of reaction mixture containing 0.25 \( \mu \)g of creatine kinase; temperature, 30\(^{\circ} \). Exchange rate \((V)\) is expressed as millimicromoles per min per \( \mu \)g of enzyme.

The basic approach used in determining the rates of isotope exchange for the creatine kinase reaction differed from that previously proposed (3, 4) and utilized by Fromm, Silverstein, and Boyer (14) and Silverstein and Boyer (16) in that the initial velocity of the incorporation of label from substrate into product was measured. The merit of this approach is that it is not necessary to rely on accurate determination of the distribution of label between substrate and product at equilibrium and it is not essential when determining the exchange patterns that the system be precisely at equilibrium. It does require simple, and preferably, rapid methods for the separation of substrate-product pairs and these are available for the separation of the nucleotides and guanidino compounds. The principle involved in the separation of creatine and phosphocreatine could also be applied to any other phosphotransferase reaction which utilizes an uncharged substrate.

The inhibition of the exchange reaction by higher concentrations of the MgATP-MgADP pair draws attention to the need for exercising caution in interpreting the meaning of exchange patterns. The inhibition of the exchange rate which develops when one like substrate-product pair is raised in concentration could indicate that these substrates were the inner pair of an ordered reaction. But such a result would also be obtained if either the substrate or the product of the pair gave rise to substrate inhibition or if inhibitory ions were introduced into the system. Similarly inhibition obtained as a result of raising the concentration of a pair of reactants which might be involved in dead end complex formation, could be due to anionic effect or substrate inhibition by one or both components.

The investigations reported here were assisted considerably by knowing the concentrations of the various ionic species which were present at equilibrium and the values for the kinetic constants associated with the various reactant species. It may well be that isotope exchange will have its greatest use as a means of confirming results obtained by the usual steady state kinetic approaches.

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