The Mechanism of the Reaction Catalysed by Adenosine Triphosphate-Creatine Phosphotransferase

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1. The forward and reverse reactions catalysed by ATP-creatine phosphotransferase have been studied kinetically at pH 8.0 in the presence and absence of products, under conditions in which the free Mg^{2+} concentration was maintained constant at 1 mM. Thus at fixed pH the reaction may be considered as being bireactant and expressed as:

2. The initial-velocity pattern in the absence of products and the product-inhibition pattern have been determined. These are consistent with a random mechanism in which all steps are in rapid equilibrium except that concerned with the inter-conversion of the central ternary complexes, and in which two dead-end complexes (enzyme-MgADP-creatine and enzyme-MgATP-phosphocreatine) are formed. The results are in accord with previous suggestions that the enzyme possesses distinct sites for the combination of the nucleotide and guanidino substrates. 3. Values have been determined for the Michaelis and dissociation constants involved in the combination of each substrate with various enzyme forms. Although these values cannot be regarded as absolute, they appear to indicate that the presence of one substrate on the enzyme enhances the combination of the second substrate. In addition, it would seem that in the formation of the enzyme-MgADP-creatine complex the concentration of one reactant does not affect the combination of the other. This contrasts with the formation of the enzyme-MgATP-phosphocreatine complex, where each reactant hinders the combination of the other.

There have been several indications that the mechanism of the reaction catalysed by ATP-creatine phosphotransferase (creatine kinase, EC 2.7.3.2) may be of the random rapid-equilibrium type (Cleland, 1963a). Thus the kinetic results of Morrison, O'Sullivan & Ogston (1961) and Morrison & O'Sullivan (1965) were in accord with the idea of rapid and independent binding of $MgADP^-$ and phosphocreatine to the enzyme. In addition, the thermodynamic studies of Kuby, Mahowald & Noltmann (1962) indicated that creatine kinase possesses distinct binding sites for the nucleotide and guanidino substrates, and the agreement between the kinetically and thermodynamically determined constants for the various enzyme-substrate complexes led Kuby & Noltmann (1962) to conclude 'that a quasi-equilibrium mechanism exists for ATP-creatine transphosphorylase'. Similar conclusions have been reached by Samuels, Nihei & Noda (1961) and Nihei, Noda & Morales (1961).

The kinetic investigations referred to above have been restricted to the measurement of initial velocities in the presence of substrates only and this approach has limitations with respect to the determination of reaction mechanisms. The advantage of measuring initial velocities in the presence of one of the reaction products has been pointed out by a number of authors including Alberty (1958) and Cleland (1963a). This procedure enables a distinction to be made between mechanisms that have the same initial-velocity equation, by virtue of the fact that the complete rate equations are different.

The only product-inhibition studies that have been made with creatine kinase are those reported by Kuby, Noda & Lardy (1954b), who found that $MgADP^-$ was a competitive inhibitor with respect to $MgATP^{2-}$ and a non-competitive inhibitor with respect to creatine. [The article by Kuby & Noltmann (1962) should be consulted for a reanalysis of this work.] However, no definite conclusions could be reached about the reaction mechanisms since

such a product-inhibition pattern is consistent with an ordered mechanism, or a rapid-equilibrium random mechanism with one dead-end complex (Cleland, 1963a), or with a rapid-equilibrium random mechanism with two dead-end complexes.

The present paper reports the results of experiments with creatine kinase in which each of the reactants has been used as a product inhibitor in relation to the two substrates under conditions where the concentration of Mg^{2+} has been held constant. The inhibition pattern so obtained suggests that the enzyme possesses two sites, one for the nucleotide and the other for the guanidino substrate, and catalyses a reaction for which all the steps are in rapid equilibrium except for that concerned in the interconversion of the central complexes. Further, they indicate that the two dead-end complexes, enzyme-MgADP-creatine and enzyme-MgATP-phosphocreatine, can form and that the presence of one substrate on the enzyme can influence the combination of the second substrate.

EXPERIMENTAL

Materials

Chemicals. Creatine (British Drug Houses Ltd., Poole, Dorset) was twice recrystallized from water, washed with ethanol and dried in air. Phosphocreatine was prepared by the method of Ennor & Stocken (1948) as modified by Peanasky, Kuby & Lardy (1957). Solutions of the latter were treated with Chelex 100 (Na^+ form; 200–400 mesh), obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., as described by Morrison & O'Sullivan (1965). The free creatine content was about 0.03 mole/100 moles of phosphocreatine. The sodium salts of ADP and ATP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. ADP was purified by the method of Morrison *et al.* (1961), and ATP by the procedure outlined by Berger (1956) except that three recrystallizations were carried out at 2°. The purified nucleotides showed only one spot that absorbed ultraviolet light after chromatography in isobutyric acid-aq. NH_3 (sp.gr. 0.88)–water (66:1:33, by vol.). However, enzymic analysis of ATP (see the Methods section) showed that this material contained 0.8 mole of ADP/100 moles. Stock solutions of ATP (25.0 mM) and ADP (10.0 mM) were adjusted to pH 7.6 with 0.5 N-NaOH and stored at -10° . The concentrations of both nucleotides were determined by measurement at 259 $m\mu$ (Bock, Ling, Morell & Lipton, 1956).

N-Ethylmorpholine (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was purified as described by Morrison *et al.* (1961), and triethanolamine (puriss), obtained from Fluka A.-G., Buchs SG, Switzerland, was used without further purification. Stock aqueous solutions of both compounds were prepared by weight and adjusted to the required pH with 5 N-HCl (microanalytical reagent; British Drug Houses Ltd.). Aqueous solutions of $MgCl_2 \cdot 6H_2O$ (analytical reagent; British Drug Houses Ltd.) were standardized as described by Morrison *et al.* (1961). Solutions of EDTA (British Drug Houses Ltd.) were neutralized with N-NaOH and filtered. The trisodium salt of phospho-

enolpyruvate was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., the sodium salts of NAD and NADH₂ from Sigma Chemical Co. and D-glucose from British Drug Houses Ltd.

Enzymes. Crystalline ATP-D-hexose 6-phosphotransferase (hexokinase, EC 2.7.1.1) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and D-glucose 6-phosphate-NADP oxidoreductase (glucose 6-phosphate dehydrogenase, EC 1.1.1.49) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Crystalline preparations of muscle ATP-pyruvate phosphotransferase (pyruvate kinase, EC 2.7.1.40) and L-lactate-NAD oxidoreductase (lactate dehydrogenase, EC 1.1.1.27) were purchased from California Corp. for Biochemical Research. The method of Kuby, Noda & Lardy (1954a) was used to isolate creatine kinase in crystalline form. Stock solutions of the enzyme were prepared and stored as described by Morrison *et al.* (1961).

Methods

Measurement of creatine-kinase activity. Reaction mixtures contained, in a total volume of 1.0 ml.: buffer (pH 8.0), 0.1 M, EDTA, 0.01 M, substrates (and in some cases also a product) at the concentrations indicated and sufficient $MgCl_2$ to maintain the concentration of Mg^{2+} at 1 mM. After the addition of the components, the tubes were kept in ice and before the addition of enzyme were incubated for 3 min. at 30°. The amount of enzyme added corresponded to 1.08 μ g. of protein for the forward and 0.54 μ g. of protein for the reverse reaction. All experiments were run for at least two time-periods (between 0.5 and 2 min.) to ensure that initial velocities were being measured, and the reaction was stopped in a manner depending on which product was to be estimated. All extinction measurements were made with a Shimadzu spectrophotometer.

N-Ethylmorpholine-HCl buffer was used for studies of the reverse reaction and replaced by triethanolamine-HCl buffer for studies of the forward reaction, since the former buffer interferes with the estimation of inorganic orthophosphate. Similar values for the kinetic constants of the reverse reaction were obtained with the two buffers. It was important that tubes containing ATP were not allowed to remain for long periods in ice as there was some hydrolysis of this compound. Dilutions of the enzyme were made in 1 mM-buffer (pH 8.0) containing EDTA (0.01 mM) (cf. O'Sullivan & Morrison, 1963).

Calculation of substrate concentrations. The concentrations of total magnesium and total nucleotide to give the required concentrations of $MgATP^{2-}$, $MgADP^-$ and phosphocreatine, while maintaining the concentration of Mg^{2+} at 1 mM, were calculated as described by Morrison *et al.* (1961), by using the appropriate apparent stability constants of the magnesium complexes. The values for $MgATP^{2-}$ and $MgADP^-$ at pH 8.0 and an ionic strength of 0.1 were taken to be 70000 and 4000 M^{-1} , respectively. Allowance was also made for the formation of Mg-phosphocreatine, which was considered inert, by using an apparent stability constant for this complex under the same conditions of 12 M^{-1} (cf. O'Sullivan & Perrin, 1964; Morrison & O'Sullivan, 1965).

The Mg^{2+} concentration was held constant at the relatively low value of 1 mM since it was shown by Morrison & O'Sullivan (1965) that higher concentrations not only inhibit the reaction, but also affect the combination of

phosphocreatine with the enzyme. One possible disadvantage of maintaining Mg^{2+} at 1 mM is that, at the highest concentration of $MgADP^-$ used (0.4 mM), the concentration of ADP^{3-} is about 0.1 mM, which may be compared with the K_i value for ADP^{3-} of 0.2–0.3 mM. However, it appears that this concentration of ADP^{3-} does not have any significant effect on the results (see the Results section and also Morrison & O'Sullivan, 1965).

Determination of protein. This was carried out by the method of Gornall, Bardawill & David (1949), with crystalline bovine serum albumin as the standard.

Estimation of reaction products. (a) Creatine. The reaction was stopped and the release of creatine from phosphocreatine determined as described by Morrison *et al.* (1961). Tubes containing all the components except enzyme were also incubated and treated in a similar fashion so as to allow for the presence of creatine in phosphocreatine. The inability to obtain phosphocreatine free of creatine limited the useful range of concentration of this compound that could be used. The contamination was small (0.03 mole/100 moles), but, because of the sensitivity of the assay procedure, relatively high blanks were obtained. A sensitive procedure was required because of the relatively low K_m value for $MgADP^-$, which permitted only a small release of creatine over the linear part of the reaction. Under the assay conditions, 0.1 μ mole of creatine gave an extinction value of 0.650.

(b) Phosphocreatine. This was estimated as inorganic orthophosphate after hydrolysis in the presence of acid molybdate and the method used was a slight modification of that described by Kuby *et al.* (1954b). The reaction was stopped by the addition of 0.5 ml. of acid molybdate solution [1.65% (w/v) ammonium molybdate in 15% (v/v) $HClO_4$] and immediately after was added 0.1 ml. of aminonaphtholsulphonic acid reducing reagent (King, 1932). The extinction of the resulting blue solution was determined at 750 $m\mu$ after 30 min. Under these conditions, 0.1 μ mole of phosphocreatine gave an extinction value of 0.255 when measured in a cell of 1 cm. light-path.

Since ATP is also hydrolysed under the assay conditions and as the forward reaction is linear for only a small percentage conversion of the substrates, owing to product inhibition by $MgADP^-$ and ADP^{3-} , the method is relatively insensitive. Thus it was necessary to determine the reaction velocity by carrying out the reaction for five time-periods between 0.5 and 1.5 min. No graphical evidence for non-linearity was obtained, but the points usually did not fit precisely on a straight line. An IBM computer was then used to fit a least-squares plot to the five points and the slope of this plot was taken as a measure of the amount of phosphocreatine formed in 1 min. The hydrolysis of ATP was found to be dependent, not only on its concentration, but also on the concentration of creatine and magnesium.

(c) ADP. The reaction was stopped by adding sufficient 1.0N-HCl (approx. 0.1 ml.) to bring the pH to 2.0. After 2 min., at which time the enzyme had been completely inactivated, an equivalent amount of 1.0N-NaOH was added to return the pH to 8.0. ADP was estimated by means of the coupled reactions catalysed by pyruvate kinase and lactate dehydrogenase, by using a modification of the procedure of Bücher & Pfeleiderer (1955). The following additions were made to each reaction mixture so as to give the indicated concentrations in a total volume of 1.8 ml.: $MgSO_4$,

8 mM; KCl, 75 mM; phosphoenolpyruvate, 7.8 mM; $NADH_2$, 0.225 mM. Pyruvate kinase and lactate dehydrogenase (5 μ g. of each) were then added and the amount of $NADH_2$ oxidized was measured at 340 $m\mu$ after 30 min., when the reaction was complete. Blank tubes contained all components except creatine kinase. Under these conditions, 0.1 μ mole of ADP causes a decrease in extinction of 0.346 when determined in a cell of 1 cm. light-path.

(d) ATP. The reaction was stopped by the same method as that used when ADP was to be determined. ATP was estimated enzymically by using the coupled reactions catalysed by hexokinase and glucose 6-phosphate dehydrogenase. To each reaction mixture, the following additions were made so as to give the indicated concentrations in a total volume of 1.3 ml.: glucose, 27 mM; NADP, 0.3 mM. Hexokinase and glucose 6-phosphate dehydrogenase (10 μ g. of each) were then added and the reduction of NADP was measured at 340 $m\mu$ after 10 min. (At this time, the reduction of NADP due to ATP was complete, but if the reaction were allowed to proceed for longer periods erroneous values were obtained because of the oxidation of glucose that occurred at different rates in the blank and control tubes.) Blanks contained all components except enzyme. Under the assay conditions, 0.1 μ mole of ATP caused an increase in extinction of 0.478 when measured in a cell of 1 cm. light-path.

Analysis of results. The linearity of all plots was checked graphically before the data were analysed with the appropriate computer programme of Cleland (1963c). The lines of the primary plots were fitted with the results obtained from analysis with the HYPER programme. The values for intercept or slope or both from this programme were further analysed with the LINE programme and the results used to fit the lines of the secondary plots. The kinetic constants reported in the Tables, together with their standard errors, were obtained by analysis of the data of the primary plots with the SEQUEN, COMP or NONCOMP programmes. All calculations were made with an IBM 1620 computer.

Values for the various kinetic constants could not be obtained accurately from tertiary plots because the limitations of the assay procedures did not permit of the use of a wide range of concentrations of the non-variable substrate. Hence it was necessary to resort to calculation. The weakness of this method is that it is necessary to substitute a value for the constant associated with the non-variable substrate. However, this difficulty has been minimized by utilizing values from the initial-velocity studies in the absence of product that appeared to be reasonably reliable.

Weighted mean values and their standard errors (S.E.M.) were calculated according to the formulae:

$$\text{Weighted mean of } x \text{ values} = \frac{\sum w_i x_i}{\sum w_i}$$

$$\text{and S.E.M. of weighted mean value} = \frac{1}{\sqrt{\sum w_i}}$$

$$\text{where } w = \frac{1}{[\text{S.E.M.}(x_i)]^2}$$

THEORY

To compare the values obtained for the various kinetic constants from studies of the reaction in each direction, experiments were carried out at 30° and pH 8.0. The use of this pH has the additional advantage that both ATP and

Scheme 1.

ADP can be considered to exist in solution in their fully ionized forms. Thus it follows that, in the presence of Mg^{2+} , the only Mg -nucleotide complexes that need to be considered are MgATP^{2-} and MgADP^- . Since it has been shown that these complexes can function as substrates for creatine kinase (cf. Kuby & Noltmann, 1962) and that the guanidino substrates react as non-complex species, then conditions may be adjusted so that at a fixed pH the reaction becomes bireactant, namely:

It will be assumed now, and justified later by the experimental results, that the reaction mechanism is of the random type that can be represented as shown in Scheme 1, where A, B, P, Q and E represent MgADP^- , phosphocreatine, creatine, MgATP^{2-} and enzyme respectively. Further, it will be assumed that the interconversion of the central complexes, EAB and EPQ, is the slowest step of the reaction, that rapid equilibrium is established at all other steps and that, in addition, the dead-end complexes EAP and EBQ can be formed by the reactions:

It should be noted that K_{ia} , K_{ib} , K_{ip} and K_{iq} represent dissociation constants for the reaction of the free enzyme with A, B, P, and Q, respectively, and the Michaelis constants K_a , K_b , K_p and K_q are dissociation constants for the reaction of A, B, P and Q with EB, EA, EQ and EP respectively. K_{ia} , K_{ib} , K_{ip} and K_{iq} are also dissociation constants, but for the reaction of A, B, P and Q with different forms of the enzyme, namely EP, EQ, EA and EB respectively.

The complete rate equation for the above reaction may be written as:

$$v = \frac{V_1[A][B] - \frac{V_2[P][Q]K_{ia}K_b}{K_pK_{iq}}}{K_{ia}K_b + K_b[A] + K_a[B] + [AB] + \frac{K_{ia}K_b[A][P]}{K_{ip}K_{ia}} + \frac{K_{ia}K_b[P]}{K_{ip}} + \frac{K_{ia}K_b[Q]}{K_{iq}} + \frac{K_{ia}K_b[P][Q]}{K_pK_{iq}} + \frac{K_{ia}K_b[B][Q]}{K_{iq}K_{ib}}}$$

where V_2 and V_1 represent the maximum velocities of the reaction in the forward and reverse directions respectively. From the equilibrium assumption, it follows that $K_{ia}K_b = K_aK_{ib}$ and that $K_{ip}K_q = K_pK_{iq}$. Since the complexes EAP and EBQ may each be formed in two ways, the relationships $K_{ia}K_{ip} = K_{ia}K_{ip}$ and $K_{ib}K_{iq} = K_{ib}K_{iq}$ are also obtained.

The complete rate equation may be modified by setting $[P] = [Q] = 0$ or $[A] = [B] = 0$ so as to obtain the initial-velocity equation in the absence of products for the reverse and forward reactions.

The equations so obtained with A, B, P and Q as the variable substrates are:

Reverse reaction:

$$\frac{1}{v} = \frac{K_a}{V_1} \left(\frac{K_{ib}}{[B]} + 1 \right) \frac{1}{[A]} + \frac{1}{V_1} \left(\frac{K_b}{[B]} + 1 \right) \quad (1)$$

$$= \frac{K_b}{V_1} \left(\frac{K_{ia}}{[A]} + 1 \right) \frac{1}{[B]} + \frac{1}{V_1} \left(\frac{K_a}{[A]} + 1 \right) \quad (2)$$

Forward reaction:

$$\frac{1}{v} = \frac{K_p}{V_2} \left(\frac{K_{iq}}{[Q]} + 1 \right) \frac{1}{[P]} + \frac{1}{V_2} \left(\frac{K_q}{[Q]} + 1 \right) \quad (3)$$

$$= \frac{K_q}{V_2} \left(\frac{K_{ip}}{[P]} + 1 \right) \frac{1}{[Q]} + \frac{1}{V_2} \left(\frac{K_p}{[P]} + 1 \right) \quad (4)$$

To predict the type of inhibition pattern to be expected, the complete rate equation was modified by setting the concentration of the product that was not present equal to zero. The resulting equations may then be rearranged, as shown below, according to the substrate that is being varied.

Reverse reaction:

(i) MgATP^{2-} (Q) as product inhibitor; A and B as variable substrates:

$$\frac{1}{v} = \frac{K_a}{V_1} \left(\left(1 + \frac{[Q]}{K_{iq}} \right) + \frac{K_{ib}}{[B]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right) \frac{1}{[A]} + \frac{1}{V_1} \left(1 + \frac{K_b}{[B]} \right) \quad (5)$$

$$\frac{1}{v} = \frac{K_b}{V_1} \left(1 + \frac{K_{ia}}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right) \frac{1}{[B]} + \frac{1}{V_1} \left(1 + \frac{K_a}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right) \quad (6)$$

(ii) Creatine (P) as product inhibitor; A and B as variable substrates:

$$\frac{1}{v} = \frac{K_a}{V_1} \left(1 + \frac{K_{ib}}{[B]} \left(1 + \frac{[P]}{K_{ip}} \right) \right) \frac{1}{[A]} + \frac{1}{V_1} \left(1 + \frac{K_b}{[B]} \left(1 + \frac{[P]}{K_{ip}} \right) \right) \quad (7)$$

$$\frac{1}{v} = \frac{K_b}{V_1} \left(\left(1 + \frac{[P]}{K_{ip}} \right) + \frac{K_{ia}}{[A]} \left(1 + \frac{[P]}{K_{ip}} \right) \right) \frac{1}{[B]} + \frac{1}{V_1} \left(1 + \frac{K_a}{[A]} \right) \quad (8)$$

Table 1. *Product-inhibition pattern to be expected for the reaction catalysed by creatine kinase on the basis that the mechanism is rapid-equilibrium random with two dead-end complexes*

A non-competitive product inhibitor may affect the slopes and vertical intercepts of a double-reciprocal plot to different extents. Hence the family of straight lines obtained at different concentrations of the inhibitor will intersect at a point to the left of the ordinate and this point may be above, on or below the abscissa.

(a) Reverse reaction

Product inhibitor

MgATP²⁻
Creatine

Variable substrate ... MgADP⁻
Competitive
Non-competitive

Phosphocreatine
Non-competitive
Competitive

(b) Forward reaction

Product inhibitor

MgADP⁻
Phosphocreatine

Variable substrate ... MgATP²⁻
Competitive
Non-competitive

Creatine
Non-competitive
Competitive

Forward reaction:

(i) MgADP⁻ (A) as product inhibitor; P and Q as variable substrates:

$$\frac{1}{v} = \frac{K_p}{V_2} \left(1 + \frac{K_{iq}}{[Q]} \left(1 + \frac{[A]}{K_{ia}} \right) \right) \frac{1}{[P]} + \frac{1}{V_2} \left(1 + \frac{K_q}{[Q]} \left(1 + \frac{[A]}{K_{ia}} \right) \right) \quad (9)$$

$$\frac{1}{v} = \frac{K_q}{V_2} \left(\left(1 + \frac{[A]}{K_{ia}} \right) + \frac{K_{ip}}{[P]} \left(1 + \frac{[A]}{K_{ia}} \right) \right) \frac{1}{[Q]} + \frac{1}{V_2} \left(1 + \frac{K_p}{[P]} \right) \quad (10)$$

(ii) Phosphocreatine (B) as product inhibitor; P and Q as variable substrates:

$$\frac{1}{v} = \frac{K_p}{V_2} \left(\left(1 + \frac{[B]}{K_{ib}} \right) + \frac{K_{iq}}{[Q]} \left(1 + \frac{[B]}{K_{ib}} \right) \right) \frac{1}{[P]} + \frac{1}{V_2} \left(1 + \frac{K_q}{[Q]} \right) \quad (11)$$

$$\frac{1}{v} = \frac{K_q}{V_2} \left(1 + \frac{K_{ip}}{[P]} \left(1 + \frac{[B]}{K_{ib}} \right) \right) \frac{1}{[Q]} + \frac{1}{V_2} \left(1 + \frac{[B]}{K_{ib}} \right) \quad (12)$$

Values for the various kinetic constants were determined as described in the legends of the Tables. The type of inhibition pattern to be expected on the basis of the above formulations is set out in Table 1, and the agreement between theory and experimental results is illustrated below.

RESULTS

Initial-velocity studies in the absence of product

The results illustrated in Figs. 1 and 2 show that double-reciprocal plots are linear, that the lines of each graph have a common point of intersection above the abscissa and that, within experimental error, the vertical co-ordinates of the cross-over points of Figs. 1(a) and 1(b) are equal, as also are those of Figs. 2(a) and 2(b). It follows then that the reaction mechanism is sequential, so that both substrates react with the enzyme before either product dissociates, and may be of the ordered Theorell-Chance or rapid-equilibrium random type (Cleland, 1963a), since all these mechanisms have the same form of initial-velocity equation.

Secondary plots of the slopes and vertical intercepts of Figs. 1 and 2 were linear, as shown in Figs. 3 and 4 respectively. The kinetic constants obtained from the experimental data are recorded in Table 2,

Fig. 1. (a) Effect of MgADP⁻ concentration on the initial velocity of the reverse reaction with phosphocreatine as the variable substrate. The concentrations of MgADP⁻ were: ○, 0.10 mM; ▲, 0.13 mM; ●, 0.2 mM; △, 0.4 mM. (b) Effect of phosphocreatine concentration on the initial velocity of the reverse reaction with MgADP⁻ as the variable substrate. The concentrations of phosphocreatine were: ■, 4 mM; ○, 5 mM; ▲, 6.7 mM; ●, 10 mM; △, 20 mM. Velocity is expressed as μ moles of creatine/ μ g. of creatine kinase/min.

Fig. 2. (a) Effect of creatine concentration on the initial velocity of the forward reaction with MgATP^{2-} as the variable substrate. The concentrations of creatine were: $\circ$, 8 mm; $\blacktriangle$, 10 mm; $\bullet$, 13.3 mm; $\triangle$, 30 mm. (b) Effect of MgATP^{2-} concentration on the initial velocity of the forward reaction with creatine as the variable substrate. The concentrations of MgATP^{2-} were: $\blacksquare$, 0.8 mm; $\circ$, 1.3 mm; $\blacktriangle$, 1.0 mm; $\bullet$, 2.0 mm; $\triangle$, 4.0 mm. Velocities are means from a number of experiments and are expressed as $\mu\text{moles of phosphocreatine}/\mu\text{g. of creatine kinase/min.}$

and these values have been used in the later calculations. The maximum velocity of the reaction was found to be $0.111 \pm 0.006 \mu\text{mole}/\mu\text{g. of enzyme/min.}$ for the forward reaction (V_2) and $0.204 \pm 0.005 \mu\text{mole}/\mu\text{g. of enzyme/min.}$ for the reverse reaction (V_1) at pH 8.0 and 30° . It is with respect to the maximum velocity of the reaction in each direction and the nature of the double-reciprocal plot for the reverse reaction that the results differ from those of Nihei *et al.* (1961). The values of the Michaelis constants for MgADP^- and phosphocreatine (Table 2) are also significantly different from those reported by these authors.

Fig. 3. Secondary plots of the slopes (Δ) and vertical intercepts ($\circ$) of Fig. 1 against the reciprocal of the concentration of (a) MgADP^- (from Fig. 1a) or (b) phosphocreatine (from Fig. 1b).

In the above calculations, the concentration of ADP^{3-} , which is a competitive inhibitor of the reaction with respect to MgADP^- , was considered to be negligible so that the values given for all the kinetic constants (Table 2) were taken as being true values. If, however, this assumption is not made, then the initial-velocity equation becomes:

$$v = \frac{V_1[A][B]}{(K_{ia}K_i + K_a[B]) \left(1 + \frac{[I]}{K_i}\right) + K_b[A] + [A][B]}$$

where $[I]$ and K_i respectively represent the concentration of ADP^{3-} and the dissociation constant for the enzyme-ADP or enzyme-ADP-phosphocreatine complex. From this formulation, it is clear that the values for K_a and K_{ia} , as obtained from plots similar to those of Fig. 3, would be only apparent and that true values must be calculated by using the K_i value for, and the concentration of, ADP^{3-} (cf. Table 3). The apparent concentration of the latter, as well as that of MgADP^- , will vary according to the stability constant used for MgADP^- . It was decided to determine the variation of K_a and

Fig. 4. Secondary plots of the slopes (Δ) and vertical intercepts ($\bullet$) of Fig. 2 against the reciprocal of the concentration of (a) creatine (from Fig. 2a) or (b) MgATP^{2-} (from Fig. 2b).

Table 2. Values of the Michaelis and dissociation constants for the various forms of creatine kinase as determined from initial-velocity studies in the absence of product

The values for the constants associated with the reverse reaction are the weighted means of the constants that were obtained by analysis of four sets of data, including that of Fig. 1, with the SEQUEN computer programme of Cleland (1963c). Those for the reverse reaction were obtained in a similar manner from the data of Fig. 2. All data were obtained at pH8.0 and 30°.

Reverse reaction		Forward reaction	
Kinetic constant	Value (mm)	Kinetic constant	Value (mm)
K_a	0.05 ± 0.01	K_p	6.1 ± 1.0
K_{ia}	0.17 ± 0.02	K_{tp}	15.6 ± 4.9
K_b	2.9 ± 0.3	K_q	0.48 ± 0.10
K_{ib}	8.6 ± 1.3	K_{iq}	1.2 ± 0.3

K_{ia} as a function of the value chosen for this constant, with and without correction for the ADP^{3-} concentration.

The results (Table 3) show that there is a small variation in the values for K_a and K_{ia} which, in general, is decreased when allowance is made for the concentration of ADP^{3-} . The difference between the corrected and uncorrected values for these constants, by using a stability constant of 4000M^{-1} for MgADP^- , was not considered to be sufficiently large to warrant the use of corrected values in the later calculations. Also, as predicted by theory, the values for K_b and K_{ib} , as well as the K_{ia}/K_a ratio, are invariant. The latter values clearly indicate that the lines of a plot, such as shown in Fig. 1(b), cut at a point above the abscissa.

Similar calculations were not made for the forward reaction. Because of the high apparent stability constant for MgATP^{2-} , only very low concentrations of ATP^{4-} were ever present and this species is virtually without inhibitory effect.

Initial-velocity studies in the presence of various products

Reverse reaction. (i) MgATP^{2-} (Q) as product inhibitor. The competitive inhibition of the reaction by MgATP^{2-} in relation to MgADP^- (Fig. 5) and the non-competitive inhibition with respect to phosphocreatine (Fig. 6) are as predicted by eqns. (5) and (6). Comparison of the apparent K_m values for MgADP^- and phosphocreatine at zero concentration of MgATP^{2-} and different concentrations of the non-variable substrate (not shown) indicated that, in this type of experiment, it is difficult to draw firm conclusions about the influence of the concentration of one substrate on the K_m value for the other (cf. Fig. 1 and Morrison & O'Sullivan, 1965).

From the secondary plots of the slopes of the lines of Fig. 5 (as well as of other plots not shown) against the concentration of MgATP^{2-} (Fig. 7), the horizontal intercepts, which would give an apparent K_{iq} value equal to

$$K_{iq}\{1 + ([B]/K_b)\}/\{1 + ([B]/K_{ib})\}$$

increase with the concentration of phosphocreatine. Thus K_{ib} cannot be equal to K_b and it also follows that K_{iq} does not equal K_{ia} because of the relationship between these four constants (see the Theory section). Calculated values for K_{iq} are given in Table 4.

Secondary plots of the slopes and vertical intercepts of Fig. 6 (and of other plots not shown) against the concentration of MgATP^{2-} (Fig. 8) indicate that these secondary plots are linear and that the apparent values for both K_{iq} and K_{ia} vary with the concentration of MgADP^- . The calculated values for these two constants as well as values for K_{ib} are given in Table 5; the K_{iq} value is higher than that reported in Table 4.

Table 3. *Effect of the stability constant value for MgADP⁻ on the kinetic constants for the reverse reaction*

The concentrations of MgADP⁻, ADP³⁻ and free phosphocreatine were calculated from the total concentrations of magnesium, ADP and phosphocreatine on an IBM 1620 computer with a programme specifically written for this purpose. The kinetic constants were determined as described in Fig. 1 by using the same four sets of initial-velocity data and the calculated concentrations of MgADP⁻ and phosphocreatine. The values given in parentheses were obtained by allowing for the formation of enzyme-ADP and enzyme-ADP-phosphocreatine, which were considered to be dead-end complexes, and calculated from the relationships:

$$K_a = \frac{\text{app. } K_a}{1 - R \left(\frac{\text{app. } K_a}{K_i} \right)} \quad \text{and} \quad K_{ia} = \frac{\text{app. } K_{ia}}{1 - R \left(\frac{\text{app. } K_{ia}}{K_i} \right)}$$

where R represents the $[\text{ADP}^{3-}]/[\text{MgADP}^{-}]$ ratio and K_i for ADP³⁻ was taken to be 0.25 mM.

Stability constant for MgADP ⁻ (M ⁻¹)	K_a (mM)	K_b (mM)	K_{ia} (mM)	K_{ib} (mM)	K_{ia}/K_a
3000	0.052 ± 0.005 (0.056)	3.09 ± 0.27	0.157 ± 0.021 (0.199)	8.63 ± 1.28	3.00 ± 0.70
4000	0.055 ± 0.005 (0.058)	2.87 ± 0.26	0.168 ± 0.023 (0.202)	8.62 ± 1.27	3.02 ± 0.70
5000	0.057 ± 0.006 (0.060)	2.87 ± 0.26	0.175 ± 0.024 (0.204)	8.65 ± 1.27	3.02 ± 0.70
0	0.069 ± 0.007	2.90 ± 0.27	0.210 ± 0.029	8.75 ± 1.29	3.02 ± 0.70

Fig. 5. Product inhibition of the reverse reaction by MgATP²⁻ with MgADP⁻ as the variable substrate, with the concentration of phosphocreatine held constant at 5 mM. The concentrations of MgATP²⁻ were: Δ , none; $\bullet$, 1 mM; $\blacktriangle$, 2 mM; $\circ$, 3 mM. Velocity is expressed as μ moles of creatine/ μ g. of creatine kinase/min.

Fig. 6. Product inhibition of the reverse reaction by MgATP²⁻ with phosphocreatine as the variable substrate, with the concentration of MgADP⁻ held constant at 0.4 mM. The concentrations of MgATP²⁻ were: Δ , none; $\bullet$, 2 mM; $\blacktriangle$, 4 mM; $\circ$, 6 mM. Velocity is expressed as μ moles of creatine/ μ g. of creatine kinase/min.

Fig. 7. Secondary plots of the slopes of Fig. 5 and similar graphs obtained at different fixed concentrations of phosphocreatine against the concentration of MgATP²⁻. The concentrations of phosphocreatine were: $\blacksquare$, 5 mM; $\bullet$, 10 mM; $\blacktriangle$, 15 mM.

(ii) Creatine as product inhibitor. The non-competitive inhibition by creatine with MgADP⁻ as the variable substrate (Fig. 9) is in accord with eqn. (7). It was not possible to obtain a definite intersection point (which would give the value of K_{ia}) for plots with the phosphocreatine concentra-

tion held constant at 5, 10 and 15 mM, although the results tended to suggest that this point may fall below the abscissa. The difficulty is probably due to the relatively small changes in the slopes of the lines that occur with increasing concentrations of creatine, but it was not possible to use higher concentrations of this compound because of its limited solubility. For this reason, secondary plots of the slopes of the lines do not give satisfactory results and are not shown. However, since analysis of primary data by means of a NONCOMP computer programme gave values for K_{ip} , these have been

Table 4. Values for K_{iq} as determined from the results of the competitive inhibition by MgATP^{2-} in relation to MgADP^-

Each apparent K_{iq} value is a weighted mean of the values determined from the results of a number of experiments, including that shown in Fig. 5, with the COMP computer programme of Cleland (1963c). K_{iq} was calculated from the relationship:

$$\text{app. } K_{iq} = K_{iq} \left(\frac{1 + \frac{[\text{B}]}{K_{ib}}}{1 + \frac{[\text{B}]}{K_{ib}}} \right)$$

where K_{ib} and K_{lb} were taken to be 8.6 mM (Table 2) and 21 mM (Table 5) respectively.

Concn. of phosphocreatine (mM)	No. of experiments	app. K_{iq} (mM)	K_{iq} (mM)
5.0	3	0.87 ± 0.03	0.68
10.0	2	1.01 ± 0.05	0.69
15.0	3	1.22 ± 0.06	0.76
Average value	...	...	0.71

Fig. 8. Secondary plots of the slopes (a) and vertical intercepts (b) of Fig. 6 and similar graphs obtained at different fixed concentrations of MgADP^- against the concentration of MgATP^{2-} . The concentrations of MgADP^- were: $\circ$, 0.1 mM; $\blacktriangle$, 0.2 mM; $\triangle$, 0.4 mM.

Table 5. Values of K_{iq} and K_{Iq} as determined from the results of the non-competitive inhibition by MgATP^{2-} with respect to phosphocreatine

Each apparent value for K_{iq} and K_{Iq} is a weighted mean of the values obtained from a number of experiments, including that illustrated in Fig. 6, with the NONCOMP computer programme of Cleland (1963c). Calculations were made from the relationships:

$$\text{app. } K_{iq} = K_{iq} \left(1 + \frac{[\text{A}]}{K_{ia}} \right) \quad \text{and} \quad \text{app. } K_{Iq} = K_{Iq} \left(1 + \frac{[\text{A}]}{K_a} \right)$$

where K_{ia} and K_a were taken to be 0.17 mM and 0.05 mM respectively (Table 2). A modified NONCOMP programme was used to determine the horizontal co-ordinate of the intersection point of each plot, which gives an estimate of the value for K_{lb} .

Concn. of MgADP^- (mM)	No. of experiments	app. K_{iq} (mM)	K_{iq} (mM)	app. K_{Iq} (mM)	K_{Iq} (mM)	K_{lb} (mM)
0.1	1	2.42 ± 0.39	1.45	8.77 ± 2.06	2.92	16.2 ± 5.3
0.2	2	2.94 ± 0.31	1.35	15.90 ± 3.70	3.18	25.2 ± 7.4
0.4	2	3.88 ± 0.38	1.16	20.30 ± 3.09	2.26	20.4 ± 4.1
Average values	...	...	...	...	2.79	20.6

Fig. 9. Product inhibition of the reverse reaction by creatine with MgADP^- as the variable substrate with the concentration of phosphocreatine held constant at 5 mm. The concentrations of creatine were: Δ , none; $\bullet$, 10 mm; $\blacktriangle$, 20 mm; $\circ$, 30 mm. Velocity is expressed as $\mu\text{moles of ATP}/\mu\text{g. of creatine kinase/min.}$

Fig. 10. Secondary plot of the vertical intercepts of Fig. 9 and similar graphs obtained at different fixed concentrations of phosphocreatine against the concentration of creatine. The concentrations of phosphocreatine were: $\blacktriangle$, 5 mm; $\bullet$, 10 mm; Δ , 15 mm.

Table 6. Values for K_{ip} and K_{I_p} as determined from the non-competitive inhibition by creatine in relation to MgADP^-

Each apparent value for K_{ip} or K_{I_p} is a weighted mean of the values from a number of experiments, including that shown in Fig. 9, and was obtained by computer analysis with the NONCOMP programme of Cleland (1963c). Calculations were made from the relationships:

$$\text{app. } K_{ip} = K_{ip} \left(1 + \frac{[B]}{K_b} \right)$$

and

$$\text{app. } K_{I_p} = K_{I_p} \left(1 + \frac{[B]}{K_b} \right)$$

by using values of 8.6 mm and 2.9 mm for K_{ib} and K_b respectively (Table 2).

Concn. of phosphocreatine (mm)	No. of experiments	app. K_{ip} (mm)	K_{ip} (mm)	app. K_{I_p} (mm)	K_{I_p} (mm)
5.0	5	36 ± 5	23	20 ± 1	7.3
10.0	5	28 ± 4	13	44 ± 2	9.8
15.0	3	54 ± 11	20	40 ± 3	6.4
Average values	...	...	19	...	8

included in Table 6, although they cannot be regarded as being reliable.

Secondary plots of the vertical intercepts of Fig. 9 and of primary plots with different fixed concentrations of phosphocreatine against the concentration of creatine (Fig. 10) were linear and showed that the apparent value for K_{I_p} was a function of the phosphocreatine concentration. Calculation gave values for K_{I_p} (Table 6) that are similar to those for K_{ip} obtained from the

Fig. 11. Product inhibition of the reverse reaction by creatine with phosphocreatine as the variable substrate, with the concentration of MgADP^- held constant at 0.2 mm. The concentrations of creatine were: Δ , none; $\bullet$, 10 mm; $\blacktriangle$, 20 mm; $\circ$, 30 mm. Velocity is expressed as $\mu\text{moles of ATP}/\mu\text{g. of creatine kinase/min.}$

results of the competitive inhibition by creatine with respect to phosphocreatine (see below).

The results illustrated in Fig. 11 appear to indicate that creatine is a competitive inhibitor in relation to phosphocreatine (cf. eqn. 8). Similar results were obtained with the MgADP^- concentration held constant at 0.1 mm. Secondary plots of the slopes of the lines of these graphs against creatine concentration are linear (Fig. 12) and give apparent values for K_{ip} equal to

$$K_{ip} \{ 1 + ([A]/K_{ia}) \} / \{ 1 + ([A]/K_{ia}) \}$$

Fig. 12. Secondary plot of the slopes of Fig. 11 and a similar graph obtained with MgADP⁻ at a concentration of 0.1 mM against the concentration of creatine. The concentrations of MgADP⁻ were: O, 0.1 mM; ●, 0.2 mM.

Fig. 13. (a) Product inhibition of the forward reaction by MgADP⁻ with creatine as the variable substrate with the concentration of MgATP²⁻ held constant at 2 mM. The concentrations of MgADP⁻ were: O, none; ●, 0.1 mM; Δ, 0.3 mM. (b) Secondary plot of the slopes (O) and intercepts (●) of Fig. 13(a) against the concentration of MgADP⁻. Velocities are means from a number of experiments and are expressed as μ moles of phosphocreatine/ μ g. of creatine kinase/min.

Since there is little variation in the apparent values with the concentration of MgADP⁻, it may be concluded that K_{ia} is approximately equal to K_{is} . Computer analysis of the data of the primary plots gave apparent K_{ip} values of 9.5 ± 0.6 mM and 8.6 ± 0.5 mM with MgADP⁻ at concentrations of 0.1 and 0.2 mM respectively. On the assumption that K_{ia} does equal K_{is} , the value of K_{ip} would be about 9 mM, which is lower than the value recorded in Table 6.

Forward reaction. (i) MgADP⁻ (A) as product inhibitor. Eqns. (9) and (10) are in agreement with the experimental results illustrated in Figs. 13 and 14. Thus MgADP⁻ is a non-competitive inhibitor with creatine as the variable substrate and a competitive inhibitor with MgATP²⁻ as the variable substrate. From the intersection point of Fig. 13(a) an inaccurate K_{ip} value of 16.5 ± 16.5 mM was obtained and this may be compared with a value

Fig. 14. (a) Product inhibition of the forward reaction by MgADP⁻ with MgATP²⁻ as the variable substrate, with the concentration of creatine held constant at 30 mM. The concentrations of MgADP⁻ were: Δ, none; ●, 0.1 mM; ▲, 0.2 mM; O, 0.3 mM. (b) Secondary plot of the slopes of Fig. 14(a) against the concentration of MgADP⁻. Velocities are means from a number of experiments and are expressed as μ moles of phosphocreatine/ μ g. of creatine kinase/min.

of about 8 mm as reported in Table 6. Secondary plots of the slopes and vertical intercepts of Fig. 13(a) against the concentration of MgADP⁻ (Fig. 13b) were linear. Computer analysis of the primary data gave values of 0.36 ± 0.22 mm for apparent K_{ia} and 0.63 ± 0.34 mm for apparent K_{Ia} . Calculation from the relationships:

$$\text{app. } K_{ia} = K_{ia} \left(1 + \frac{[Q]}{K_{iq}} \right)$$

and

$$\text{app. } K_{Ia} = K_{Ia} \left(1 + \frac{[Q]}{K_q} \right)$$

Fig. 15. Product inhibition of the forward reaction by phosphocreatine with creatine as the variable substrate, with the concentration of MgATP²⁻ held constant at 2 mM. The concentrations of phosphocreatine were: Δ , none; $\bullet$, 5 mM; $\blacktriangle$, 10 mM. Velocity is expressed as μ moles of ADP/ μ g. of creatine kinase/min.

Fig. 16. Product inhibition of the forward reaction by phosphocreatine with MgATP²⁻ as the variable substrate, with the concentration of creatine held constant at 30 mM. The concentrations of phosphocreatine were: Δ , none; $\bullet$, 10 mM; $\blacktriangle$, 20 mM; $\circ$, 30 mM. Velocity is expressed as μ moles of ADP/ μ g. of creatine kinase/min.

gave values of 0.14 mm for K_{ia} and 0.12 mm for K_{Ia} when K_{iq} and K_q were taken as being 1.2 mm and 0.48 mm respectively (Table 2). These results

Fig. 17. Secondary plot of the slopes of Fig. 15 and a similar graph obtained with MgATP²⁻ at a concentration of 4 mM against the concentration of phosphocreatine. The concentrations of MgATP²⁻ were: $\bullet$, 2 mM; $\circ$, 4 mM.

Fig. 18. Secondary plot of the slopes (a) and vertical intercepts (b) of Fig. 16 and similar graphs obtained at different fixed concentrations of creatine against the concentration of phosphocreatine. The concentrations of creatine were: $\bullet$, 15 mM; $\circ$, 22.5 mM; $\blacktriangle$, 30 mM.

appear to confirm the similarity of the two values although the standard errors were high. Further, they are lower than the value given for K_{ia} in Table 2; this would be expected when no allowance is made for the contribution that ADP^{3-} would make towards the inhibition.

The points of a secondary plot of the slopes of Fig. 14(a) against the concentration of MgADP^- (Fig. 14b) are somewhat scattered, but the plot was considered to be linear. Analysis of the primary data of Fig. 14(a) gave an apparent K_{ia} value, equal to $K_{ia}\{1 + ([\text{P}]/K_{ip})\}/\{1 + ([\text{P}]/K_{ip})\}$, of $0.10 \pm 0.01 \text{ mM}$. As this value is similar to the true value obtained

from the results of Fig. 13(a) (see above) it is in accord with the previous conclusion that K_{ip} is approximately equal to K_{ip} .

Studies of the inhibition by MgADP^- were not carried out at a number of concentrations of the non-variable substrate as the method used for the estimation of phosphocreatine was not sufficiently sensitive. When either of the non-variable substrates was fixed at a lower concentration, velocity measurements were inaccurate, and this was also the case with higher concentrations of MgATP^{2-} because of the relatively large amounts of inorganic orthophosphate released in acid molybdate.

(ii) Phosphocreatine (B) as product inhibitor. The results illustrated in Figs. 15 and 16 show that phosphocreatine is a competitive inhibitor with respect to creatine and a non-competitive inhibitor in relation to MgATP^{2-} , as required by eqns. (11) and (12). Further, secondary plots of the vertical intercepts or slopes or both of these plots (and of others not shown) are linear, as illustrated in Figs. 17 and 18.

The nature of the variation of apparent K_b with the concentration of MgATP^{2-} (Fig. 17) is consistent with the hyperbolic relationship shown in Table 7. However, computer analysis with the COMP programme gave values for apparent K_b that were slightly different from those indicated in Fig. 17. The former values have been used to calculate the true K_{ib} value (Table 7).

The values of 4.1 mM for K_{ib} and 14 mM for K_{ib} (Table 8) may be compared with values of 6.7 mM (Table 7) and 20.6 mM (Table 5) respectively. Values for K_{iq} , as determined from the cross-over point of plots similar to that of Fig. 16, are listed in Table 8. The average value is similar to that reported in Table 5.

Table 7. Values for K_{ib} as determined from the results of the competitive inhibition by phosphocreatine with creatine as the variable substrate

Each apparent K_{ib} value is the weighted mean of the values obtained by computer analysis of a number of experiments, including that shown in Fig. 15, with the COMP programme of Cleland (1963c). K_{ib} was determined from the relationship:

$$\text{app. } K_{ib} = K_{ib} \left(\frac{1 + \frac{[\text{Q}]}{K_{iq}}}{1 + \frac{[\text{Q}]}{K_{iq}}} \right)$$

by using values of 1.2 mM for K_{iq} (Table 2) and 3.5 mM for K_{iq} (Table 8).

Concn. of MgATP^{2-} (mM)	No. of experiments	app. K_{ib} (mM)	K_{ib} (mM)
2.0	3	8.7 ± 0.6	5.1
4.0	2	16.8 ± 1.3	8.3
Average value ...		...	6.7

Table 8. Values for K_{ib} and K_{ib} as determined from the results of the non-competitive inhibition by phosphocreatine in relation to MgATP^{2-}

Each apparent value for K_{ib} or K_{ib} is the weighted mean of the values obtained from the data of a number of experiments, including that shown in Fig. 16, by computer analysis with the NONCOMP programme of Cleland (1963c). Calculations were made from the relationships:

$$\text{app. } K_{ib} = K_{ib} \left(1 + \frac{[\text{P}]}{K_{ip}} \right) \quad \text{and} \quad \text{app. } K_{ib} = K_{ib} \left(1 + \frac{[\text{P}]}{K_{ip}} \right)$$

by taking K_{ip} and K_{ip} to be 15.6 mM and 6.1 mM respectively (Table 2). A modified NONCOMP programme was used to determine the horizontal co-ordinates of the intersection points of the plots, which gave an estimate of the value for K_{iq} .

Concn. of creatine (mM)	No. of experiments	app. K_{ib} (mM)	K_{ib} (mM)	app. K_{ib} (mM)	K_{ib} (mM)	K_{iq} (mM)
15.0	3	8.8 ± 1.2	4.5	45.8 ± 8.0	13	4.2 ± 1.0
22.5	2	9.5 ± 1.6	3.9	70.5 ± 14.9	15	3.3 ± 1.1
30.0	3	11.5 ± 1.8	3.9	78.8 ± 13.0	13	3.1 ± 0.8
Average values ...		...	4.1	...	14	3.5

Table 9. *Values for the Michaelis and dissociation constants at pH 8.0 and 30° for the reaction catalysed by creatine kinase*

Reactant	K_m (mM)	K_i (mM)	K_r (mM)
MgATP ²⁻ (Q)	0.48	0.71–1.34	2.8–3.5
MgADP ⁻ (A)	0.05	0.14–0.17	0.12
Creatine (P)	6.1	16–19	8–17
Phosphocreatine (B)	2.9	4.1–8.6	14–21

The range of values for the various kinetic constants associated with the creatine-kinase reaction are summarized in Table 9.

DISCUSSION

The results of the kinetic studies of the reaction catalysed by creatine kinase in the absence and presence of substrate are consistent with the reaction being a random one for which all the steps, except that involving the interconversion of the central complexes, are in rapid equilibrium. In addition, they suggest that the enzyme possesses distinct binding sites for the nucleotide and guanidino substrates so that the dead-end complexes enzyme-MgADP-creatine and enzyme-MgATP-phosphocreatine can form. However, the product-inhibition pattern on which the above conclusions are based is also consistent with a Theorell-Chance mechanism (Cleland, 1963a) for which either MgADP or phosphocreatine is the first reactant in the reverse sequence. But the latter possibility can be eliminated, for in a Theorell-Chance mechanism the apparent inhibitor constants obtained from all four competitive inhibitions would not vary with the concentration of the fixed substrate and it has been found that at least two constants, K_u and K_b , do vary with the concentration of phosphocreatine and MgATP²⁻ respectively. The findings of Kuby *et al.* (1962) that creatine kinase combines with each of the four substrates as determined by equilibrium dialysis and ultracentrifuge measurements also argues against a Theorell-Chance mechanism. These results are also inconsistent with the reaction having an ordered mechanism (Cleland, 1963a). In addition, the product-inhibition pattern obtained (Table 1) is not in accord with that expected for a simple ordered mechanism (cf. Cleland 1963a) or an ordered mechanism with one or two dead-end complexes. Further, the formation of dead-end complexes would give rise to non-linear primary or secondary plots or both and such non-linearity was not found. Studies of isotope exchange at equilibrium would not only distinguish between these mechanisms, but would also provide firmer evidence for the characterization of the creatine-kinase reaction as rapid-equilibrium random.

The general conclusions with respect to the mechanism of the creatine-kinase reaction are in accord with the results of other investigations. Thus, as mentioned above, they are in agreement with the thermodynamic experiments of Kuby *et al.* (1962) showing that each of the substrates can combine independently with the enzyme and with the lack of exchange between [³²P]ADP and ATP in the absence of the guanidino substrates (Noda, Nihei & Morales, 1960), which indicates that a ternary enzyme complex must be formed. Although Molnar & Lorand (1960) have reported that the above exchange can occur in the absence of creatine or phosphocreatine, this result has not been confirmed.

Initial-velocity studies of the creatine-kinase reaction have also been made by Nihei *et al.* (1961). These authors reported that at pH 6.4 the K_m values for phosphocreatine and MgADP⁻ are not affected by the concentration of MgADP⁻ and phosphocreatine respectively, whereas in the present work it has been found that at pH 8.0 the presence of one substrate on the enzyme does affect the combination of the second substrate. This difference cannot be accounted for by the concentration of free Mg²⁺, which was held constant at 1 mM, since subsequent experiments (J. F. Morrison & M. L. Uhr, unpublished work) have shown that similar results are obtained with Mg²⁺ maintained constant at 10 mM. These findings draw attention to the fact that independent binding at the two sites of an enzyme is not a prerequisite for a random mechanism and point to the possibility that the substrates induce conformational changes in this enzyme. Such changes could also be related to the formation of two dead-end complexes. In the reactions catalysed by yeast hexokinase (Fromm & Zewe, 1962) and pyruvate kinase (Reynard, Hass, Jacobsen & Boyer, 1961) the presence of one substrate on the enzyme does not influence the combination of the second substrate. However, whereas only one dead-end complex is formed with pyruvate kinase, hexokinase appears to catalyse the formation of two dead-end complexes of the type obtained with creatine kinase.

The results of the product inhibition by MgADP⁻ with respect to MgATP²⁻ and creatine are similar to those of Kuby *et al.* (1954b) in that MgADP⁻ functioned as a competitive inhibitor when the MgATP²⁻ concentration was varied and as a non-competitive inhibitor when the creatine concentration was varied. But a quantitative comparison cannot be made because of the differences in the two sets of experimental conditions and the fact that in the work of Kuby *et al.* (1954b) inhibition resulted from the presence of MgADP⁻ as well as ADP³⁻ (cf. Kuby & Noltmann, 1962). However, since both MgADP⁻ and ADP³⁻ react at the same site on the enzyme (Morrison & O'Sullivan, 1965) the presence of the two species would not alter the

inhibition pattern, although the inhibition constant so obtained would not be correct for either MgADP^- or ADP^{3-} . The uncorrected data of Kuby *et al.* (1954b) referred to above have been the subject of a number of analyses. Reiner (1959) concluded that the mechanism could not be ordered, but his conclusion was based on an erroneous assumption, as pointed out by Cleland (1963b), who showed that the reaction could be either ordered or random rapid-equilibrium with one dead-end complex. Walter & Frieden (1963) disagreed with the suggestion of Kuby *et al.* (1954b) that their results indicated that the enzyme has distinct sites for the attachment of ATP and creatine. This conclusion was based on the eightfold difference between the K_i values for ADP as obtained from the competitive and non-competitive inhibitions. They argued that these values should be the same if they represented the dissociation constant for the same enzyme-ADP complex. However, in a random rapid-equilibrium mechanism with one or two dead-end complexes, the K_i value for ADP as obtained from secondary plots of the slopes and intercepts of the non-competitive inhibition with respect to creatine are only apparent values because, in addition, there is competition between ADP and the fixed substrate ATP. Indeed, it is clear from the calculations given in the Results section that there is no discrepancy in the K_i value for MgADP^- as determined from the competitive and non-competitive inhibitions.

No attempt has been made to compare the magnitude of the kinetic constants reported in the present paper with those previously obtained. It was considered that there was little merit in doing so since the conditions under which the constants have been determined have varied with respect to pH, temperature, ionic strength and the composition of the buffer. Moreover, in many instances values for the apparent, rather than real, Michaelis constants have been given. It is emphasized that, although an endeavour has been made to obtain accurate values for the kinetic constants, there were a number of factors that mitigated against this aim. The analytical methods available were not sufficiently sensitive and were such as to require estimation of the amount of product formed at different time-periods. In addition, the sensitivity of the assays for creatine, ATP and phosphocreatine was further decreased by the presence of creatine in phosphocreatine, ADP in ATP and the hydrolysis of ATP in acid molybdate respectively. These difficulties may well be overcome by use of a pH-stat, which would give a continuous record of the reaction velocity and be independent of the blank values, but this type of apparatus was not available. The magnitude of the kinetic constants also depends to a large extent on the use of the correct apparent stability

constants for MgADP^- , MgATP^{2-} and $\text{Mg-phosphocreatine}$ under the experimental conditions, as well as on the conclusion that $\text{Mg-phosphocreatine}$ is inert. Since the apparent stability constant is a function of the ionic strength, the value should be corrected if the concentrations of the components of the reaction mixture are such as to cause significant changes in this parameter, and it may also be necessary to take into account the nature of the ions added. The somewhat formidable task of determining and correcting for the influence of various ions on the apparent stability constants of the magnesium complexes was not undertaken and may constitute one of the reasons for the failure to obtain better agreement for those constants that can be derived from different sets of results.

Since it was shown that the kinetic constants obtained from the initial-velocity data of the reverse reaction were influenced to only a small extent when the stability constant for MgADP^- was varied within the range $3000\text{--}5000\text{M}^{-1}$, and as the kinetic constants for the forward reaction, as determined from initial-velocity studies, are less subject to variation because of the high stability constant for MgATP^{2-} and the weak inhibitory effect of ATP^{4-} , the values recorded in Table 2 were used to calculate the K_{eq} of the reaction. When these, together with the values for V_1 and V_2 , were substituted into the Haldane relationship:

$$K_{eq} = \frac{V_2 K_{ia} K_b}{V_1 K_{ip} K_i}$$

and allowance was made for the H^+ concentration (because of the release of a proton in the forward reaction), then a value of 3.6×10^{-10} at pH 8.0 for K_{eq} was obtained. This is in reasonable agreement with the value of 3.0×10^{-10} as calculated by Kuby & Noltmann (1962) from the concentrations of reactants at equilibrium. [A rapid-equilibrium random mechanism is generally characterized by four Haldane relationships, but since the kinetic constants (Table 2) were determined from an overall fit to the initial-velocity data in which $K_{ia} K_b$ was made equal to $K_a K_b$ and $K_{ip} K_i$ made equal to $K_p K_i$, then, in effect, only one Haldane relationship applies.]

Although the values for the kinetic constants cannot be regarded as being absolute, the kinetically determined dissociation constants for the reaction of the various substrates with the free form of the enzyme are in agreement with the thermodynamic values of Kuby *et al.* (1962) in that the order of binding was $\text{MgADP}^- > \text{MgATP}^{2-} > \text{phosphocreatine} > \text{creatine}$. Further, it does seem that there is justification for concluding that there is little difference in the reaction of creatine with the free enzyme and with enzyme- MgADP . On the

other hand, the reaction of phosphocreatine with the enzyme is hindered by the presence of MgATP^{2-} and the reverse is also true. The similarity of the values for K_b and K_t appear to suggest that the binding of phosphocreatine to enzyme-MgATP may occur through the creatine moiety of phosphocreatine, whereas the attachment of MgATP to the enzyme is hindered by the presence of phosphocreatine.

The approach that has been used with respect to the control of the metal ion concentration differs from that used by Reynard *et al.* (1961) and Fromm & Zewe (1962) in their investigations of the mechanism of the reactions catalysed by pyruvate kinase and hexokinase. Whereas these authors added a constant amount of total magnesium, in the present work it is the concentration of Mg^{2+} that has been maintained constant by allowing for the ability of the components of the system to chelate magnesium. The disadvantages of the former procedure can be illustrated by analysis of the results of Reynard *et al.* (1961), who studied the inhibition by ATP of the pyruvate-kinase reaction in the reverse direction. Under all conditions the total concentration of magnesium was 10 mM and it was assumed that this concentration was sufficiently high to convert ADP^{3-} to MgADP^- . However, this was not so when ATP, up to concentrations of 7.5 mM, was added. At this concentration of ATP and pH 8.5, the Mg^{2+} concentration is approx. 2.5 mM and it may be calculated, by using a stability constant of 4000 M^{-1} for MgADP^- , that under these conditions there would be approx. 0.7 mM- ADP^{3-} and 1.4 mM- MgADP^- . At lower concentrations of ATP, there would be correspondingly lower concentrations of ADP^{3-} and higher concentrations of MgADP^- . It becomes apparent that the inhibition obtained on the addition of ATP could be due, not only to MgATP^{2-} , but also to ADP^{3-} and decrease in the concentration of MgADP^- . This conclusion seems to be confirmed by the fact that a secondary plot of the slopes of Fig. 3 of the paper by Reynard *et al.* (1961) against the concentration of ATP gives a parabolic-type curve. Thus the average K_t value quoted for ATP must be considered approximate.

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