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Kinetics of Coupled Enzymes. Creatine Kinase and Myosin A*

Jean Botts† and M. Judith Stone

ABSTRACT: When adenosine triphosphate is added to a two-enzyme system containing myosin A, creatine kinase, and creatine phosphate, the adenosine diphosphate produced by the myosin-catalyzed hydrolysis of adenosine triphosphate can be rapidly rephosphorylated at the expense of creatine phosphate by the action of creatine kinase. If both enzymes in the two-enzyme system are able to function as they do individually, then, under appropriate conditions, the rate of creatine production should reflect the rate of adenosine triphosphate hydrolysis. It is observed, however, that the rate of creatine production in the two-enzyme system is less than the rate of inorganic phosphate production in the corresponding myosin system. Activities in the presence and absence of added magnesium and pyrophosphate also show this discrepancy. Evidence indicates that magnesium introduced with the kinase can account for only a part of the inhibition. The remaining difference in the activities of the two systems cannot be attributed to inhibition of myosin A by creatine phosphate, to a delay in establishing the steady-state rate of regenerating adenosine triphosphate, nor to a steady-state adenosine triphosphate concentration appreciably below the total nucleotide concentration. The inhibition of enzymatic activity in the two-enzyme system seems to be attributable to a direct inhibition of myosin by creatine kinase.

In the contraction–relaxation cycle of muscle, ATP is considered to be the immediate source of chemical energy; but a major reservoir of readily available chemical energy is creatine phosphate. Creatine kinase, which catalyzes the transphosphorylating reaction in which ATP is formed from ADP and creatine phosphate, can act in tandem with myosin ATPase to constitute a coupled system catalyzing the hydrolysis of creatine phosphate (reactions i–iii). Creatine kinase has also been

$$\text{ATP} + \text{H}_2\text{O} \rightleftarrows \text{ADP} + \text{P}_i \qquad \text{(i)}$$

$$\text{ADP} + \text{creatine phosphate} \rightleftarrows \text{ATP} + \text{creatine} \qquad \text{(ii)}$$

$$\text{creatine phosphate} + \text{H}_2\text{O} \rightleftarrows \text{creatine} + \text{P}_i \qquad \text{(iii)}$$

implicated in the relaxation process either as a direct participant or as an important means of regenerating ATP utilized in the process of calcium accumulation by the sarcoplasmic reticulum (Lorand *et al.*, 1965). Myosin and creatine kinase can be extracted from muscle in comparable amounts on a molar basis. But, since these enzymes are known to be compartmented within the

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muscle fiber, it is not particularly useful to consider average concentrations. Myosin A is localized in the thick filaments of the myofibrils, and at least some creatine kinase is associated with the sarcoplasmic reticulum immediately surrounding each myofibril. In this location the kinase can readily come in contact and react with the ADP produced by the ATPase of the sarcoplasmic reticulum. Under some conditions the calcium concentrating ability of the sarcoplasmic reticulum is strongly dependent upon the efficiency of the ATP-regenerating action of the kinase (Lorand *et al.*, 1965). Whether creatine kinase is also strategically located close to myosin ATPase is not known, but the ATP requirements of the contractile process would make such an arrangement an attractive possibility. Hori (1966) has found that when fixed sections of rat skeletal muscle are incubated with ADP, creatine phosphate, calcium, and lead, lead phosphate precipitate can be found in the A bands as well as in the region of the triad cisternae of the sarcoplasmic reticulum. He points out, however, that ATP formed through creatine kinase activity might migrate to regions of ATPase activity in the A band, so that the presence of creatine kinase in the A band is not certain. (No evidence of myokinase activity in the A band was found.)

In several laboratories (*e.g.*, Nanninga and Mommaerts, 1960) a two-enzyme system involving an ATPase and a transphosphorylase has been used as a convenient way of maintaining the ATP level while hydrolysis is going on. Under appropriate conditions with a high concentration of creatine phosphate, ADP can be rapidly and almost completely converted into ATP (Noda *et al.*, 1954). In accordance with reactions i–iii, the rate of creatine production through reaction ii can then be a measure of the rate of hydrolysis of ATP in reaction i. In the two-enzyme system involving myosin A and creatine kinase there is some evidence that the enzymes can interact, and that inhibition of enzymatic activity results. Under ionic conditions in which myosin A precipitates and creatine kinase normally remains in solution, Yagi and Mase (1962) have reported that brief centrifugation sediments both enzymes. In the presence of creatine kinase, myosin ATPase activity appears to be reduced (Botts and Stone, 1960; Yagi and Mase, 1962).

The present paper is concerned with a further comparison of activities in the one- and two-enzyme systems. In particular, the validity of measurements in the two-enzyme system has been reexamined. Reactions were studied in the presence of magnesium, which activates creatine kinase and inhibits myosin ATPase, and with PP, which, in the absence of magnesium, activates myosin ATPase and inhibits creatine kinase but which, in the presence of magnesium, inhibits both enzymes. In the two-enzyme experiments described below, the creatine kinase concentration was always large relative to the myosin A concentration so as to minimize any problem of sluggish ATP regeneration. The activity in the two-enzyme system should then reflect essentially the activity of the myosin component.

Materials and Methods

Preparation of Myosin A. Myosin A was obtained by a brief (~10 min) extraction of rabbit skeletal muscle mince in phosphate-buffered KCl solution (0.6 ionic strength) at pH 6.5 or by a 2-hr extraction at pH 6.8 in the presence of ATP at an initial concentration of 2.0 mM. The enzyme was then purified by alternately lowering and raising the ionic strength in successive cycles to precipitate and redissolve the myosin A. At the beginning of the second cycle, 0.40 mM ATP was added to the redissolved protein. Reduction of the ionic strength was then carried out in two steps by first diluting to an ionic strength of 0.1–0.2 and immediately centrifuging to remove insoluble material including myosin B. The supernatant was then further diluted to precipitate the remaining myosin; after redissolving the myosin, the stepwise dilution procedure was repeated. The final myosin A solution was centrifuged at 30,000*g* for 1 hr and dialyzed against 0.5 M KCl lightly buffered with $KHCO_3$ at pH 7.

Creatine Kinase. Solutions of the crystalline enzyme were kindly supplied by Professor L. H. Noda and were prepared in accordance with the standard procedure of his laboratory.

Nucleotides. The sodium in solutions prepared from crystalline disodium ATP (Sigma) and sodium ADP (Sigma) were exchanged for potassium on an Amberlite IR-120 cation-exchange column.

Other Salts. Analytical reagent grade KCl was recrystallized from glass-distilled water after filtering the solution to remove traces of insoluble substances including iron-containing matter. Since SO_4^{2-} inhibits creatine kinase (Noda *et al.*, 1960), Mg, when present, was added in the form of magnesium acetate. Reagent grade $Na_4P_2O_7 \cdot 10H_2O$ was dissolved, filtered, and precipitated in double-distilled ethanol, redissolved in water at 80°, and allowed to recrystallize slowly. Creatine phosphate and Tris buffer (best grade) were obtained from Sigma Chemical Co. and were used without further purification.

α-Naphthol Reagent. Steam-distilled α-naphthol crystals were dried, stored in the dark, and used within a few days after distillation. Prior to addition of α-naphthol crystals to $NaHCO_3$–NaOH alkali stock (Eggleton *et al.*, 1943), purified nitrogen was bubbled for several minutes through the solution. Immediately before use, the α-naphthol crystals (2 g/100 ml of alkali stock) were added and dissolved with the help of the bubbling nitrogen. The α-naphthol solution was then maintained under a moist nitrogen atmosphere (without bubbling) and used for not more than 2 hr, usually for only 1 hr. Standard curves of $OD_{520\,m\mu}$ *vs.* [creatine] were obtained at the beginning and end of the period during which each α-naphthol solution was used. The slopes of such standard curves were generally in good agreement (within 2–3%), and the α-naphthol solution remained clear and colorless for over 2 hr. (When only 1 g of α-naphthol was used in 100 ml of alkali stock, some decline in sensitivity to creatine was noted over a 1-hr period.)

PMB[1]–Cu^{2+} Reagent. Two volumes of water plus one

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: PMB, *p*-hydroxymercuribenzoate; ANSA, aminonaphtholsulfonic acid reagent.

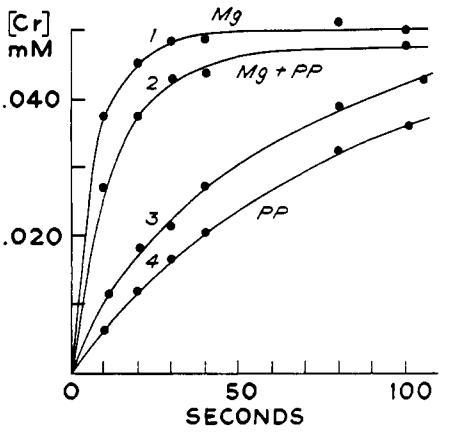

FIGURE 1: Creatine production, catalyzed by creatine kinase, accompanying the conversion of ADP into ATP in accordance with reaction ii, at pH 8.30 and 25.0°. The reaction solution contained: 0.050 mM ADP, 9.2 mM creatine phosphate, 0.167 M KCl, 0.028 M Tris, and 1.37 μM creatine kinase (added at zero time). Curve 1: 0.100 mM magnesium and no PP; curve 2: 0.100 mM magnesium and 0.113 mM PP; curve 3: no added magnesium or PP; and curve 4: no added magnesium and 0.113 mM PP.

volume of PMB solution (concentration giving optical density of 0.820 at 232 mμ $\approx 4.83 \times 10^{-5}$ M) plus one volume of 1.17 mM $CuSO_4$.

Diacetyl Reagent. Freshly prepared 1/20 dilution (by volume) of 1% stock solution in water.

Aminonaptholsulfonic Acid Reagent. Na_2SO_3 (1.2 g), $NaHSO_3$ (1.2 g), and ANSA (0.14 g) (methanol rinsed and dried) were diluted to 100 ml with water and stored in the dark.

Molybdate Reagent. Ammonium molybdate (50 g) in 200 ml of water and 300 ml of 10 M H_2SO_4 were diluted to 1 l. with water.

General Procedure in Measuring Enzymatic Activity. Reactions were generally carried out in 0.167 M KCl and 0.028 M Tris buffer at 25.0° and pH 8.30. (Measurement of the pH at the beginning and end of the reactions indicated that this pH was maintained.) Reaction solutions were continuously stirred with a magnetic stirrer. ATP solution, freshly prepared and at neutral pH, was added to the reaction solution just prior to addition of the enzyme(s). In order to avoid preliminary dilution of myosin A, a small amount of the stock (1–2%) enzyme was added directly to the reaction solution with a micrometer syringe. Normally a total of four aliquots was withdrawn from each vessel beginning 100 sec following the addition of myosin. Aliquots were spaced at 100-sec intervals for rapid reactions and at 300-sec intervals for slower reactions. For comparable conditions in the one- and two-enzyme systems, the same aliquot spacing and the same myosin concentration were used. In the one-enzyme system containing myosin, the rate of P_i production was normally measured, although in special cases the hydrogen ion production was followed instead, as noted below. In the two-enzyme system the rate of net reaction iii was followed as the rate of creatine production. In this case, myosin was added 300 sec after addition of creatine kinase to allow time for the establishing of equilibrium between ATP and ADP prior to the initiation of the ATPase reaction. Conditions were such that at equilibrium the nucleotide was essentially all in the form of ATP.

Measurement of P_i Production. Inorganic phosphate was measured by means of a slight modification of the Fiske–Subbarow method (Fiske and Subbarow, 1925). From 25 ml of reaction solution 5-ml aliquots were withdrawn at specified times, inactivated in 2 ml of 10% trichloroacetic acid, and filtered; 350 sec later 5 ml of filtrate was added to 1 ml of molybdate reagent 400 sec prior to the addition of 0.5 ml of ANSA reagent. The optical density at 750 mμ was read 700 sec following ANSA addition. (The presence of magnesium, PP, and/or ATP did not affect the slope of the phosphate standard curve, optical density *vs.* $[P_i]$.) The small amount of ATP hydrolysis occurring in the presence of acid reagents was essentially the same for all four aliquots of a given reaction solution and therefore did not affect the slope of the reaction curve (optical density *vs.* time).

Measurement of Hydrogen Ion Production. At pH 8.30 the hydrogen ion production accompanying dephosphorylation of ATP is practically equal to the P_i production. The rate of H^+ production was measured with a Radiometer recording pH-Stat at 25.0°. The reaction solution was magnetically stirred under a purified nitrogen atmosphere.

Measurement of Creatine Production. A modification of the method of Eggleton *et al.* (1943) and Ennor and Stocken (1948) was used. The enzymatic activity in each 2-ml aliquot of reaction solution was stopped at a specified time with 1 ml of PMB–Cu reagent. (Cu^{2+} completely inhibits both enzymes.) Fifty seconds later 1 ml of α-napthol reagent was added, immediately followed by 0.5 ml of diacetyl reagent. After 5-min centrifugation in a clinical centrifuge, the supernatant was carefully pipetted into a spectrophotometer cuvet (decanting stirs up the precipitate) and the optical density at 520 mμ was read 1900 sec following inactivation of the enzyme(s). The presence of magnesium and/or PP did not affect the slope of the standard curve.

Measurement of Metal Ion Concentrations. Analyses for magnesium and calcium were made in a Perkin-Elmer Model 303 atomic analyzer using solutions analogous to those in the experiments described below. Analysis for residual sodium in ATP following cation exchange on a column was made in a Beckman spectrophotometer with flame photometer attachment and was found to be negligible.

Results and Discussion

Under a variety of conditions it has been found that the enzymatic activity in the two-enzyme system is lower than that in the corresponding one-enzyme (myosin) system. In some cases the creatine kinase in the two-enzyme system can be shown to be the rate-limiting enzyme. In other instances, however, there seems to be a real discrepancy in the myosin activities of the two systems.

Under the experimental conditions used, it is known that Mg^{2+} activates creatine kinase but inhibits myosin. In well-dialyzed crystalline creatine kinase preparations such as were used in these experiments, Kuby *et al.* (1954)

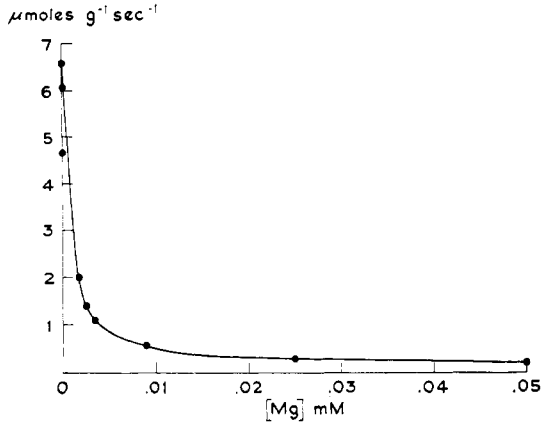

FIGURE 2: Inhibiting effect of magnesium on myosin ATPase activity at pH 8.30 and 25.0°. [KCl] = 0.167 M, [ATP] = 0.20 mM, [myosin A] range = 0.00428–0.0514 mg/ml. Measurements made in a pH-Stat.

found that the total ash of the enzyme would be sufficient for only 2 moles of magnesium/mole of creatine kinase. When residual magnesium is removed by a chelating agent, the creatine kinase activity disappears. Figure 1 shows that, even in the absence of added magnesium, the enzyme is still able to effect a fairly rapid conversion of ADP into ATP. This activity may depend, in part, on the presence of a metal ion impurity as well as on magnesium associated with creatine kinase.

In the two-enzyme system some of the magnesium originally associated with creatine kinase may be preferentially bound to myosin with a resulting inhibition of the ATPase activity. If concentrations were such that this binding appreciably reduced the level of magnesium available for kinase activation, a lowering of the activities of both enzymes could result. Since the kinase concentration in the two-enzyme experiments was always far in excess of the myosin concentration, myosin inhibition would be a more likely cause of the lowered activities. In the myosin system containing 0.0828 μM myosin, 0.080 mM ATP, and no added magnesium, addition of 10.1 μM creatine kinase (with no creatine phosphate) reduced the myosin activity to about one-third of the control value. Figure 2 shows the inhibiting effect of small amounts of added magnesium on the myosin ATPase activity in 0.167 M KCl at pH 8.30. When the concentration of added magnesium is 1.5–2.0 μM, myosin activity drops to about one-third the value obtained in the absence of added magnesium. Therefore, if about 10% of the magnesium originally associated with the creatine kinase were able to act in inhibiting myosin activity, the observed drop in activity upon addition of creatine kinase would be explained. Whether myosin can successfully compete for this tightly bound magnesium is not known.

In the presence of PP (with no added magnesium) the discrepancy between the activities in the one- and two-enzyme systems is even more pronounced. The one-enzyme (myosin) system is slightly activated whereas the two-enzyme system is further inhibited. Reducing the level of any inhibiting magnesium in the myosin system, and of kinase-activating magnesium in the two-enzyme

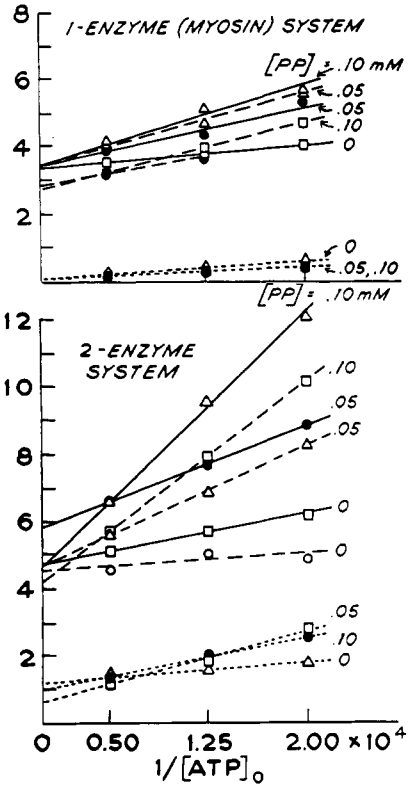

FIGURE 3: Comparison of the activities in the one- and two-enzyme systems at pH 8.30 and 25.0°, 0.167 M KCl, and 0.028 M Tris. In both systems, myosin A concentration was 0.0348 mg/ml (0.074 μM) when no magnesium was added, and 0.068 mg/ml when magnesium was added. In the two-enzyme system, the creatine kinase concentration was 0.281 mg/ml (3.46 μM) throughout. Added Mg concentrations were: 0.10 mM (——), 0.05 mM (- - - - -), and 0 mM (· · · · · ·). Each line is labeled according to the total PP concentration (millimolar). The ordinate is in $\mu moles^{-1}$ g sec.

system, would be expected to have this qualitative result; but probably such a simple explanation for the effect of PP is not altogether adequate. Although the affinity constant for magnesium binding to PP^{4-} is more than ten times that for magnesium binding to ATP^{4-} (Vasil'ev 1957; Burton, 1959), the final acid dissociation constants of the two chelators are such that, for the same total concentrations, ATP is actually better than PP as a sequestering agent for Mg at pH 8.3 (Botts *et al.*, 1966). Therefore, for concentration ranges of ATP from 5 to 20 × 10^{-5} M and of PP from 5 to 10 × 10^{-5} M, addition of PP to a solution already containing ATP at pH 8.30 would reduce the free magnesium concentration by a factor of generally not less than 0.5. Furthermore, in the two-enzyme system containing 0.074 μM myosin, 0.10 mM PP, and 0.20 mM ATP, doubling and quadrupling the creatine kinase concentration (from 3.48 μM to 6.96 and 13.9 μM) progressively lowers the rate of creatine production, which would not be the case if creatine kinase activity were the limiting factor. Figure 3 shows that, with added magnesium, PP has an inhibitory action on myosin. It is therefore possible that, in the absence of added magnesium, the inhibition of the two-enzyme system by PP is due partly to the presence of a higher level of in-

TABLE I: Myosin ATPase Activities under Various Conditions in the Presence and Absence of Creatine Phosphate.[a]

[Creatine Phosphate] (mM)	[PP] (mM)	[Mg] (mM)	v (μmoles g^{-1} sec^{-1})
0	0	0.05	0.310
0	0	0.05	0.306
11.2	0	0.05	0.346
0	0.10	0	5.69
0	0.10	0	5.75
11.2	0.10	0	5.27
0	0.10	0.05	0.212
11.2	0.10	0.05	0.207

[a] Activities were measured in the pH-Stat at 25.0° and pH 8.30. [KCl] = 0.167 M, [ATP] = 0.20 mM; no creatine kinase.

trinsic magnesium than that existing in the corresponding one-enzyme (myosin) system.

If the presence of magnesium in the two-enzyme system were solely responsible for the discrepancy in activities between the one- and two-enzymes systems, then adding a sizable amount of Mg to both systems should bring the activities to the same value. However, Figure 3 shows that even with added magnesium (at a concentration more than 14 times that of creatine kinase) the activity in the two-enzyme system is still well below the value in the corresponding system with myosin alone, and is also less than that in the myosin system containing twice this amount of added magnesium.

In attempting to explain the differences in activities in the comparable one- and two-enzyme systems, one must examine the distinguishing features of each system. The two systems have several components in common. Corresponding reaction mixtures contain the same concentrations of myosin, KCl, Tris buffer, ATP, PP, and added magnesium. The two-enzyme system contains, in addition, creatine phosphate and creatine kinase. Since creatine phosphate is present at a relatively high concentration (12.0 mM), it is necessary to ask what effect such a creatine phosphate level may have on the activity of myosin. To avoid the breakdown of creatine phosphate in the acid medium used in the Fiske–Subbarow method, the pH-Stat, measuring the rate of H^+ production, was chosen as a convenient means of comparing myosin activities in the presence and absence of creatine phosphate (Tris buffer was omitted in these reactions). Table I shows that the activities with and without creatine phosphate are not appreciably different regardless of the presence of added magnesium or PP. We may therefore assume that neither creatine phosphate nor any impurity associated with creatine phosphate has an appreciable effect on the myosin ATPase activity.

According to Kuby *et al.* (1954), creatine kinase at a concentration of 0.281 mg/ml would contain not more than 7.0 μM magnesium. In myosin preparations similar to those used here, a concentration of 0.068 mg/ml contained about 2.5 μM magnesium. Analysis of the magnesium content of a typical reaction solution shows that 0.167 M KCl, 0.20 mM ATP, and 0.028 M Tris would contribute, respectively, 0.35, 0.11, and 0.15 μM magnesium or a total of 0.61 μM magnesium. The concentrations of magnesium in PP and creatine phosphate solutions were not measured; but, since neither of these substances alone causes inhibition of myosin ATPase, the magnesium content is assumed to be low. (The calcium concentrations in KCl and ATP were found to be about the same as the magnesium concentrations; in Tris and myosin the calcium concentrations were somewhat less, about 50–60% of the magnesium concentrations.) If the total magnesium concentration in the two-enzyme system for Figure 3 (exclusive of added magnesium) can be estimated at about 10 μM, addition of 50 or 100 μM magnesium would increase by severalfold the total magnesium concentration in the reaction solution. In particular, the one-enzyme (myosin) system containing 100 μM added magnesium (giving a total of about 103 μM magnesium) would have a higher magnesium content than the two-enzyme system to which only half this concentration of magnesium has been added. Yet the activity in the one-enzyme system is significantly higher (Figure 3).

It is clear that, if myosin is added to a creatine kinase system which has been allowed to reach equilibrium concentrations of nucleotides, there will be a time lag before steady-state nucleotide levels are established. In this interval, ADP will be accumulating because initially the rate of ATP hydrolysis must exceed the rate of ATP regeneration. This fact raises two questions: (1) During the time that the two-enzyme reaction solution is being sampled, do steady-state conditions prevail, *i.e.*, is creatine kinase regenerating ATP at the same rate at which myosin is hydrolyzing it? (2) Is the steady-state concentration of ATP very nearly the same as its equilibrium level? An affirmative answer to both of these questions is implied if one assumes that the plots in Figure 3 give a valid comparison of the myosin activities in the corresponding one- and two-enzyme systems. To examine further the validity of those assumptions, three different approaches were used: (1) extending the time interval of aliquot sampling in the two-enzyme system, (2) simulating the action of myosin by a continuous addition of ADP to the creatine kinase system at a known rate, and (3) simulating the kinetics of the two-enzyme system with the analog computer.

In the first approach, the creatine kinase system (with and without added magnesium) was allowed to reach its equilibrium level of ATP prior to addition of myosin. The reaction solution was then sampled over a 45-min period instead of the usual few 100 sec. Figure 4 shows that the rate of creatine production remained constant throughout the entire time of sampling. Although there was some evidence of a short time lag in the reaction mixture containing no added magnesium, the steady state appears to be established within the first 100 sec even under these unfavorable conditions. In the reac-

tion mixture to which magnesium was added, there was no detectable lag; the linear portion of the curve following myosin addition extrapolates back essentially to the point of myosin addition. These results are in agreement with the findings of Nanninga and Mommaerts (1960) who used the luciferin–luciferase reaction to follow the concentration of free ATP in a multienzyme system containing myosin and creatine kinase. Under their experimental conditions, a steady-state concentration of ATP (91–99% of the total [ATP]) was established within 50 sec after initiation of the reactions.

To interpret the myosin activity reflected in curve a of Figure 4, it is necessary to know the actual substrate concentration, *i.e.*, it is necessary to know to what extent the steady-state concentration of ATP is perceptibly lower than the equilibrium level. One way of estimating the steady-state level of ATP under these conditions is to find the ordinate point at which the tangent (dashed line) to the left-hand portion of curve a is parallel to the steady-state rate of creatine production following myosin addition. This point would determine the ATP level at which the kinase-catalyzed formation of ATP is just balanced by the myosin-catalzyed hydrolysis of ATP. The ordinate value at the tangent point, expressible as the concentration of ATP rather than creatine, is then taken as the steady-state concentration of ATP maintained by the two enzymes under the given conditions. In applying this method, one assumes that creatine kinase is equally active in the presence and absence of myosin. Alternatively, by a method not depending upon this assumption, a lower bound for the steady-state concentration of ATP for curve a in Figure 4 can be taken as the ATP level obtained by extrapolating the right-hand, linear portion of the curve back to its ordinate value at 1000 sec (the time of myosin addition). If the initial rate of ATP hydrolysis following myosin addition is essentially the same as that ultimately established in the steady state, the ATP concentration cannot fall below the intersection of this extrapolated line and the line (indicated in the inset in Figure 4) which would represent the decline in [ATP] under these conditions if no regenerating (kinase) system were present. In the presence of the regenerating system, the actual fall in [ATP] would be less marked and might follow a time course such as that represented schematically by the dotted line in the inset. By either of the methods described here, it can be concluded that the steady-state concentration of ATP for curve a is not less than 92% of its equilibrium value and is very probably above 96%. Therefore, even in the absence of added magnesium, creatine kinase can maintain the ATP concentration at very nearly the total nucleotide concentration. The differences in the results for the one- and two-enzyme systems shown in Figure 3 cannot, therefore, be attributed to improper assignment of the abscissa values for the two-enzyme system.

In the second approach for establishing the validity of the assumptions made in the two-enzyme system, the ability of creatine kinase to react with very low levels of ADP in the presence of a much higher concentration of ATP was tested in another way. The action of myosin in forming ADP was simulated by adding very small amounts of ADP solution at a nearly continuous rate

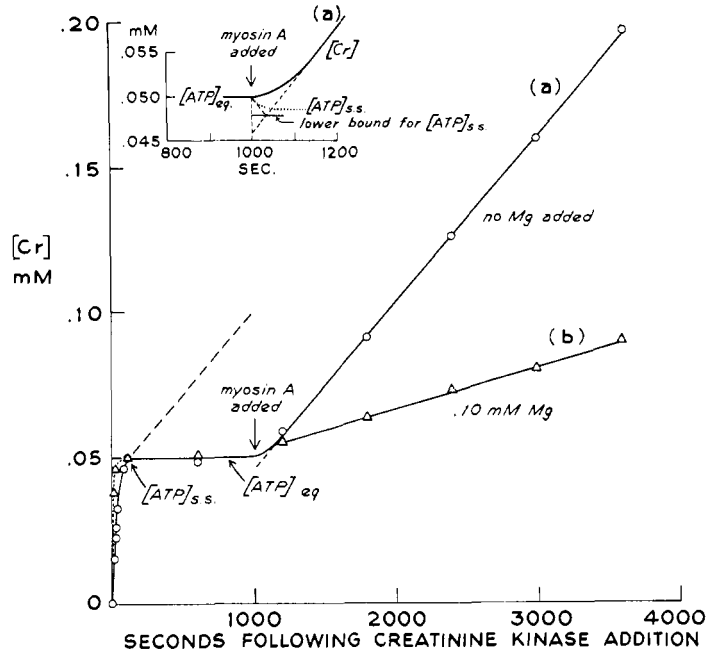

FIGURE 4: Establishment of the steady-state concentration of ATP, $[ATP]_{ss}$. Initial conditions: 0.012 M creatine phosphate, 0.05 mM ADP, 0.167 M KCl, 0.028 M Tris, pH 8.30, 25.0°. Curve a: no magnesium added; curve b: 0.10 mM magnesium. At zero time creatine kinase was added (1.37 μM) and the rate of creatine production was followed in each case until the equilibrium level of ATP, $[ATP]_{equil}$, was established. At 1000 sec, myosin A was added (curve a: 0.073 μM; curve b: 0.145 μM) and the ATPase activity, as reflected by creatine production, was followed for the next 2600 sec. Curve a appeared to experience a slight time lag in establishing $[ATP]_{ss}$. The inset shows a schematic representation of the time course of creatine production as $[ATP]_{equil} \rightarrow [ATP]_{ss}$. The significance of the dashed line, drawn parallel to the right-hand portion of curve a and tangent to the left-hand portion of this curve, is explained in the text.

to the creatine kinase reaction system containing a known concentration of ATP. Aliquots were withdrawn from the reaction vessel at the same time intervals as for the regular activity runs and were analyzed in the usual way for creatine. In these experiments ADP solution was added to the well-stirred reaction solution at a fixed initial rate by means of a hand-operated micrometer syringe. The volume increase due to ADP addition was negligible (less than 1%). The rate of ADP addition was decreased appropriately to compensate for each aliquot withdrawn from the reaction vessel. In this way the simulated rate of ADP production was maintained at 0.179 μmole $l.^{-1}$ sec^{-1}. Table II summarizes the results. It is seen that at the higher ATP concentration (1.00 mM) the rate of creatine appearance was 12–20% lower than the rate of ADP addition. However, for 0.100 mM ATP which is in the range of the ATP concentrations used in the activity measurements illustrated in Figure 3 (0.050–0.200 mM ATP), the creatine kinase was found to keep pace very well except possibly in the presence of magnesium and PP together where the rate of creatine appearance was some 8% lower than the rate of ADP addition. (Note, however, that the creatine kinase concentration used here was only half that used in the experiments of Figure 3.) As a further control, these

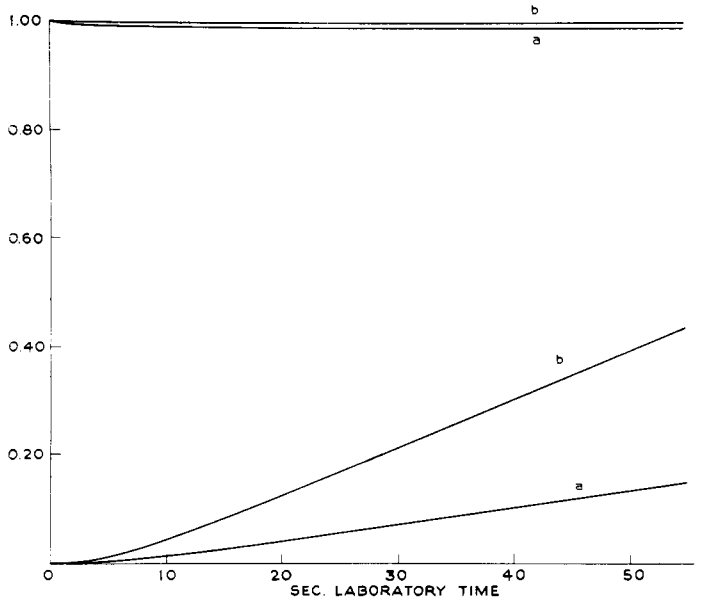

FIGURE 5: Simulation of $[ATP]/[A]_0$ (upper curves) and $[creatine]/v_{max}^{kinase}$ (lower curves) for the two-enzyme system in the presence of 10^{-4} M $MgCl_2$. The appropriate reaction constants for these conditions are listed in the text of the Appendix. $[A]_0$: (a) 0.05 mM, (b) 0.08 mM.

experiments were repeated for the higher ATP concentration, but with creatine itself substituted for ADP in the micrometer syringe. In this case, the measured rates of creatine appearance matched the rate of creatine addition reasonably well except, again, when both magnesium and PP were present. The "equilibrium" creatine concentration remained at only 83% of the total added, 500 sec following cessation of creatine addition to the reaction vessel containing magnesium plus PP. But, even if the steady-state concentration of ATP were as much as 10–20% lower than the total nucleotide concentration, this would still not account for the fact that generally the two-enzyme system at the highest nucleotide concentration used, 0.200 mM, showed a lower activity than did the one-enzyme (myosin) system with only 0.050 mM ATP (Figure 3).

The third approach to the study of the two-enzyme system was that of analog computer simulation of the enzymatic activities under various conditions. Details of this part of the investigation are given in the Appendix. It was assumed that each enzyme in the two-enzyme system is able to function as it does in the absence of the other enzyme. The two main results of this study were that, even under the most adverse conditions assumed, the steady state was essentially achieved within 30 sec and the steady-state level of ATP did not fall below 89% of its equilibrium value. In the more typical cases, the time lag before the steady state was achieved was negligible, and the steady-state ATP concentration was within 97% of its equilibrium value.

By various means of estimating the steady-state ATP level in the two-enzyme system, one concludes that this value is very nearly equal to the total nucleotide concentration. However, since a fairly high concentration of creatine kinase was used in the two-enzyme system,

TABLE II: Creatine Kinase Activity in the Presence and Absence of Magnesium and/or PP When ADP (or Creatine) Was Added at a Continuous Rate (0.179 μmole $l.^{-1}$ sec^{-1}) to Simulate the Action of Myosin ATPase (see text).[a]

		Observed Rate of Creatine Appearance on Addition of 0.179 μmole $l.^{-1}$ sec^{-1} of:[b]		
		ADP		Creatine
[Mg] (mM)	[PP] (mM)	ATP = 10^{-4} M	ATP = 10^{-3} M	ATP = 10^{-3} M
0	0	0.170	0.156	0.179
0	0.10	0.179	0.163	0.180
0.05	0	0.177	0.163	0.170
0.05	0.10	0.165	0.143	0.150

[a] Reactions were carried out at 25.0° and pH 8.30; [creatine kinase] = 1.73 μM, [creatine phosphate] = 0.012 M, [KCl] = 0.167 M, [Tris] = 0.028 M. [b] μmole $l.^{-1}$ sec^{-1}.

we might also consider the possibility that this enzyme can bind sufficient nucleotide to reduce the concentration of free ATP available to myosin. If this were the case, then increasing the ATP concentration should tend to overcome this discrepancy. Although it cannot be said with certainty that the curves in Figure 3 are linear near the ordinate as depicted here, there is no real indication that the corresponding curves for the two systems are tending to converge at higher ATP concentrations.

The results above can be summarized as follows. In the absence of added magnesium, the cause of reduced activity in the two-enzyme system (as compared with the one-enzyme myosin system) is ambiguous. However, the lowered activity cannot be attributed to a failure of the creatine kinase to keep pace with the myosin nor to an inhibition of myosin due to the presence of a high concentration of creatine phosphate in the two-enzyme system. There is a possibility that some of the magnesium associated with creatine kinase is transferred to myosin with a resulting inhibition of the myosin ATPase activity. But, in the presence of added magnesium, a discrepancy in activities between the one- and two-enzyme systems persists. Since the magnesium concentration is then essentially the same in the two systems, the residual difference in activities cannot be attributed to magnesium inhibition of myosin. Nor can it be attributed to a failure of the creatine kinase in the two-enzyme system to regenerate ATP rapidly and maintain a steady-state concentration very close to its equilibrium level, since creatine kinase is activated by magnesium. The lowering of the myosin activity in the two-enzyme system would seem to be most readily explainable in terms of a direct interaction between the two enzyme molecules, as proposed previously (Botts and Stone, 1960; Yagi and Mase, 1962).

Appendix

The computer simulation of the enzyme kinetics was

designed and carried out by Mr. David McKay and Professor Manuel F. Morales.

Rate equations for catalysis of ATP hydrolysis by myosin and for phosphate transfer (from creatine phosphate to ADP) by creatine kinase are available (*e.g.*, Ouellet *et al.*, 1952; Morrison and James, 1965) provided that the concentrations of the enzymatic intermediates can be assumed to be stationary in time. If the two enzyme systems are present in the same solution and interact only in competition for common substrates and if the variations in substrate concentrations are not so fast as to disturb the stationarity of the enzyme intermediates, then entirely in terms of ATP (where [ATP] + [ADP] = $[A]_0$, a constant) we may write a differential equation asserting that the net rate of ATP accumulation is the difference between the steady-state rate at which ATP (or creatine) is generated by the kinase system and the steady-state rate at which ATP is destroyed by the ATPase system. The procedure is, first, to integrate the differential equation and obtain [ATP] as a function of time, [ATP] (t). Knowledge of [ATP] (t) permits one to write an expression for the rate at which creatine or ATP is generated, *i.e.*, the differential equation for [creatine]; another integration then gives [creatine] (t). Knowledge of [ATP](t) also gives a notion of how long it takes for [ATP](t) to settle down to its "steady" value, $[ATP]_{ss}$, or for [creatine](t) to become linear. If the ATPase rate is measured from the linear phase of [creatine](t), such a rate corresponds to an [ATP] which is somewhat less than $[A]_0$, and one must ascertain the validity of assuming it to be $[A]_0$.

An Electronic Associates TR-48 analog computer with plotter was used in representing the kinetics of the two enzyme systems acting in concert. The "system constants" required from the analysis were taken from the present work and from the values reported by Morrison and James (1965) under experimental conditions (pH 8.0, 30°) similar to those used here. These investigators found the dissociation constant to be 0.05 mM for the reaction E·ADP·creatine phosphate $\rightleftarrows$ E·creatine phosphate + ADP and 2.79 mM for the reaction E·creatine phosphate·ATP $\rightleftarrows$ E·creatine phosphate + ATP. From the present study, the Michaelis constants, K_m^{myosin}, for myosin ATPase in 10^{-4} M $MgCl_2$ and in 10^{-4} M PP were found to be 0.35 and 0.10 mM, respectively. Under these same two conditions, with enzyme concentrations the same as for Figure 3, v_{max} values for myosin (v_{max}^{myosin}) were 0.01 and 0.272 μM sec^{-1}, respectively; the corresponding values for creatine kinase (v_{max}^{kinase}) were 5.58 and 3.16 μM sec^{-1}. The values programmed for $[A]_0$ were those used experimentally (0.05, 0.08, and 0.20 mM).

The time-dependent variables whose behavior was actually simulated were [ATP](t)/$[A]_0$ and [creatine](t)/v_{max}^{kinase}. "Good" behavior, in the sense that $[A]_0$ approximates $[ATP]_{ss}$ and that transient effects are short-lived, is associated with small values for the ratios $R_v \equiv v_{max}^{myosin}/v_{max}^{kinase}$ and $r \equiv K_m^{myosin}/[A]_0$. The simulation shows that in most experiments the behavior is indeed good, *i.e.*, $[ATP]_{ss}$ is indistinguishable from $[A]_0$ and, within 20–30 sec after mixing, the rate of creatine production faithfully represents the rate of ATP hydrolysis (Figure 5). The "worst" conditions ensue when 10^{-4} M PP is activating myosin and inhibiting the kinase and when low values of $[A]_0$ are employed; R_v and r are then relatively large. Even under such conditions there is only about a 10% difference between $[A]_0$ and the computed value for $[ATP]_{ss}$, and there is only about a 40-sec lag in establishing a steady-state rate of creatine production (Figure 6).

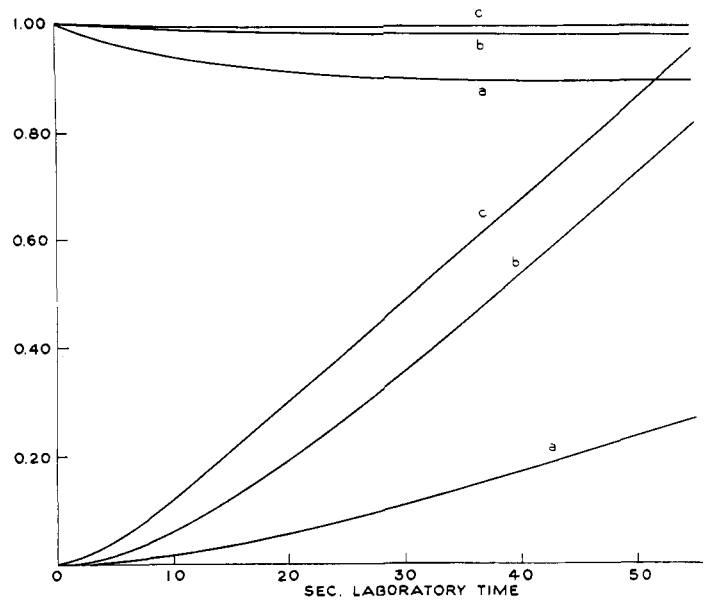

FIGURE 6: Simulation of [ATP]/$[A]_0$ (upper curves) and ($^1/_3$) [creatine]/v_{max}^{kinase} (lower curves) for the two-enzyme system in the presence of 10^{-4} M PP. The appropriate reaction constants for these conditions are listed in the text of the Appendix. $[A]_0$: (a) 0.05 mM, (b) 0.08 mM, and (c) 0.20 mM.

Thus, if the two enzyme systems are assumed not to interact except in competition for common substrates, the analog computer simulation of the enzyme kinetics indicates that the rate of creatine production in the two-enzyme experiments should accurately reflect the ATPase activity of myosin in less than 1 min following initiation of the reaction. Under experimental conditions such as were used here, [ATP] would be maintained at very nearly the total nucleotide concentration, $[A]_0$.

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The Degradation of Tritiated Dihydrosphingosine in the Intact Rat*

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ABSTRACT: Tritiated dihydrosphingosine, prepared by the catalytic reduction of sphingosine with tritium gas in the presence of platinum on charcoal, was administered by intravenous injection to mature rats. The animals were sacrificed after periods of time which varied from 15 to 90 min and the total lipids of the livers were isolated. Between 18 and 26% of the injected radioactivity were present in the liver lipids and were shown to be about equally divided between the long-chain base groups and the fatty acid groups. The largest part of the tritium of the fatty acid fraction was present in palmitic acid. Both the amount and the intramolecular distribution of the radioactivity in the palmitic acid led to the conclusion that this compound was formed directly from the injected dihydrosphingosine by reactions which resulted in the metabolic removal of carbon atoms 1 and 2 from the lipid base molecule. Evidence was obtained that sphingosine and phytosphingosine are probably not intermediates in the enzymatic degradation of dihydrosphingosine.

Studies on the distribution of sphingosine and dihydrosphingosine in various animal tissues which were analyzed according to the method described by Schmidt *et al.* (1966) revealed that only a small percentage of the total lipid bases in rat liver and kidney could be accounted for as dihydrosphingosine. Since dihydrosphingosine is believed to be a precursor of sphingosine, we attempted to obtain data pertinent to the mechanism by which the characteristic proportions of sphingosine to dihydrosphingosine in tissues are maintained by injecting tritium-labeled dihydrosphingosine into rats and measuring the appearance of tritium in sphingosine. In the course of these experiments, it was noted that in addition to the tritium labeling of both dihydrosphingosine- and sphingosine-containing sphingolipids, a considerable amount of radioactivity was recovered in the fatty acids of rat liver following the injection of [^{3}H]-dihydrosphingosine.

The conversion of phytosphingosine into pentadecanoic acid has been reported by Barenholz and Gatt (1967) who injected rats with labeled phytosphingosine obtained by growing cultures of *Hansenula ciferri* in the presence of ^{3}H- or ^{14}C-labeled precursors. While the present study was in progress, Stoffel and his collaborators (1967a–c) reported observations concerning the *in vivo* metabolism of radioactively labeled lipid bases which were prepared by organic synthesis. Their data show that in contrast to phytosphingosine, both sphingosine and dihydrosphingosine are degraded to palmitic acid and ethanolamine. In the present communication, observations providing an independent confirmation of the conclusions of Stoffel *et al.* (Stoffel and Sticht, 1967a,b; Stoffel *et al.*, 1967) as well as additional information which eliminates sphingosine as a required intermediate in the biological degradation of dihydrosphingosine are described.

Experimental Section

Solvents. All solvents used in these experiments were

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