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A Study of the Kinetics of the Reactions Catalysed by Arginine Phosphokinase

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Some general properties of arginine phosphokinase (APK), which catalyses the approach to equilibrium of the reaction phosphoarginine + adenosine diphosphate $\rightleftharpoons$ arginine + adenosine triphosphate have been described in a previous communication (Morrison, Griffiths & Ennor, 1957). Although the properties of the enzyme have also been studied to some extent by Lohmann (1935; 1936) and Soreni, Dvornikova & Degtyar (1949), no detailed study has been made of the kinetics of the reaction. This has been undertaken in the present work, which was also designed to elucidate the role of Mg^{2+} ions in the mechanism of the reaction. It has previously been reported that Mg^{2+} ions activate APK (Morrison *et al.* 1957), and it is well known that Mg^{2+} ions activate other phosphate-transferring enzymes (Lehninger, 1950). However, little work has been directed towards an understanding of the role of Mg^{2+} ions in phosphate-transfer reactions. The results reported in this communication indicate that Mg^{2+} ions react with APK to form an active Mg -APK complex which is responsible for catalysing the reaction as expressed above. In this paper the following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PA, phosphoarginine; APK, arginine phosphokinase.

EXPERIMENTAL

The materials and experimental methods, together with the preparation of the enzyme used in the present investigation, have been fully described in a previous publication (Morrison *et al.* 1957). The APK preparation had a purity of 67–80%. The enzyme was stored at 5° in the presence of an equal volume of glycerol and diluted with 0.05M *N*-ethylmorpholine of the appropriate pH before addition to the test system. Although the preparation was contaminated by myokinase, the activity of this enzyme was not detectable at the low enzyme concentrations and short reaction periods used for the kinetic studies. Even with the larger enzyme concentrations and longer incubation periods used for the equilibrium experiments, it appeared that the presence of myokinase did not influence the equilibrium values. The detailed descriptions of the experimental conditions are given in the legends to the figures and tables. All experiments have been carried out at 5° for reasons previously explained (Morrison *et al.* 1957). The K_m values represent a measure of the substrate concentration required for half-maximum velocity and are not necessarily true dissociation constants of the enzyme-substrate complexes.

The reactions, $PA + ADP \rightarrow \text{arginine} + ATP$ and $\text{arginine} + ATP \rightarrow PA + ADP$ are taken as the forward and reverse reactions respectively.

RESULTS

Effect of bivalent cations on the reaction velocity. The effect of Mg^{2+} ion concentration on the reaction velocity of the reverse and forward reactions is

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shown in Figs. 1 and 2 respectively. In the reverse reaction (Fig. 1) it is seen that there is a relationship between the initial velocity and the ATP and Mg^{2+} ion concentrations. Thus for each concentration of ATP, a maximum velocity is reached when the Mg^{2+} ion concentration is approximately equal to that of ATP. The maximum velocity is also dependent upon the concentration of ATP. When the Mg^{2+} ions:ATP ratio exceeded 1:1, as a result of increasing the Mg^{2+} ion concentration, there was no decrease in the reaction velocity.

In the forward reaction at pH 7.2 (Fig. 2) maximum enzymic activity at the three levels of ADP was attained when the concentration of Mg^{2+} ions was greater than that of ADP. There was no relationship between the concentrations of ADP and Mg^{2+} ions as was the case in the reverse reaction, for

Fig. 1. Effect of Mg^{2+} ion concentration on the initial velocity of the reverse reaction in the presence of various concentrations of ATP. The reaction mixture contained arginine ($10^{-2}M$), *N*-ethylmorpholine ($0.06M$, pH 8.4) and APK ($25 \mu g.$ of protein/ml.). Total volume, 1.0 ml.; temp. 5° .

Fig. 2. Effect of Mg^{2+} ion concentration on the initial velocity of the forward reaction in the presence of various concentrations of ADP. The reaction mixture contained PA ($5 mM$), *N*-ethylmorpholine ($0.06M$, pH 7.2) and APK ($1 \mu g.$ of protein/ml.). Total volume, 1.0 ml.; temp. 5° .

the ratio of Mg :ADP at maximum enzymic activity varied from 3 to 8 according to the ADP concentration. When the Mg^{2+} ion concentration was increased considerably above that required for maximum velocity, there was a fall in the reaction rate which was dependent upon the ADP concentration; the fall was greater with lower concentrations of ADP.

Dissociation constants of APK for Mg^{2+} and Mn^{2+} ions. In order to elucidate the role of bivalent metals in the action of APK, the Mg^{2+} ion concentration was varied while the other components of

Fig. 3. Effect of Mg^{2+} ion concentration on the initial velocity of the forward reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 2. Concn. of ADP: mm . $v = \mu moles/mg./min.$

Fig. 4. Effect of Mg^{2+} and Mn^{2+} ion concentrations on the initial velocity of the reverse reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 1. Concn. of ATP: $5 mM$. $v = \mu moles/mg./min.$

both the forward and reverse reaction systems were held constant. When the reciprocals of the initial reaction velocities were plotted against the reciprocals of the Mg^{2+} ion concentrations according to the method of Lineweaver & Burk (1934), straight lines were obtained. Fig. 3 shows the results obtained for the forward reaction at pH 7.2 and 7.9, and Fig. 4 shows the results obtained for the reverse reaction at pH 8.4 and 7.9. Similar results were obtained when the Mn^{2+} ion concentration in the reverse reaction system was varied (Fig. 4).

The above results conform to a Michaelis-Menten relation, i.e. to the equation $v = V_x/(K_x + x)$, where v is initial reaction velocity, V is maximum reaction velocity, x is cation concentration and K_x is the dissociation constant of the metal(Me)-APK complex formed according to the equation:

They are, therefore, consistent with the hypothesis that one Mg^{2+} or Mn^{2+} ion combines with each active centre of the enzyme to form the active complex.

The calculated dissociation constants and V_{max} values are listed in Table 1. When the concentrations of ADP and ATP are sufficiently high to saturate the enzyme, namely, $10^{-3}M$ and $5 \times 10^{-3}M$ respectively, it will be seen that the dissociation constant of the Mg -APK complex at pH 7.9 differs as determined in the forward and reverse reactions. Moreover, the dissociation constant of the Mg -APK complex, as determined in the forward reaction, was increased as a result of a fivefold increase of the ADP concentration. The dissociation constant of the metal-APK complex is the same for both Mg^{2+} and Mn^{2+} ions, although the V_{max} value is greater in the presence of Mn^{2+} ions. This finding is in agreement with the previous finding (Morrison *et al.* 1957) that the activation of APK by Mn^{2+} ions is greater than by Mg^{2+} ions.

Table 1. Dissociation constants of Mg -APK and Mn -APK complexes

Conditions for the forward reaction were as described in Fig. 3; those for the reverse reaction as described in Fig. 4.

Metal	pH	Concn. of ADP (mm)	Dissociation constant (mm)	V_{max} (μ moles/mg./min.)
Forward reaction				
Mg^{2+}	7.2	1	0.54	57.0
	7.9	1	0.43	45.5
	7.9	5	1.0	—
Reverse reaction ([ATP]=5 mm)				
Mg^{2+}	7.9	—	0.87	7.5
	8.4	—	1.5	10.7
Mn^{2+}	8.4	—	1.5	12.5

Effect of substrate concentrations on the reaction velocity. (a) Forward reaction. The effect of ADP on the reaction velocity of the forward reaction at pH 7.2 was determined under two sets of experimental conditions. In the first, all the components of the reaction system, including Mg^{2+} ions, were held constant and the ADP concentration was varied. The results plotted according to the method of Lineweaver & Burk (1934) are shown in Fig. 5. With a Mg^{2+} ion concentration of $5 \times 10^{-3}M$, a Michaelis-Menten relation is obtained over a limited range of ADP concentrations, i.e. between 10^{-3} and $4 \times 10^{-4}M$. Within this range, the K_m value of APK for ADP was calculated to be $2.4 \times 10^{-4}M$. As would be expected from the results shown in Fig. 2, when the ADP concentration falls below $4 \times 10^{-4}M$ in the presence of $5 \times 10^{-3}M$ Mg^{2+} ions the reaction velocity decreases more markedly, so that the reciprocal values are higher than those required for a straight-line plot. Thus at the lower concentration of ADP, the Michaelis-Menten relation does not hold. When the Mg^{2+} ion concentration was varied simultaneously with the ADP concentration so as to maintain a Mg^{2+} ion:ADP ratio of 5:1, a Michaelis-Menten relation was obtained over the whole range of ADP concentrations investigated (Fig. 5). Under these conditions, the K_m value was calculated to be $3 \times 10^{-4}M$. This value is similar to that obtained above.

The effect of PA concentration on the reaction velocity in the presence of Mg^{2+} ions at a concentration of $5 \times 10^{-3}M$ is also shown in Fig. 5. It was

Fig. 5. Effect of PA and ADP concentrations on the initial velocity of the forward reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained *N*-ethylmorpholine (0.06M, pH 7.2) and APK (1 μ g. of protein/ml.). O, Variable substrate: PA, Mg^{2+} ions (5 mm), ADP (2 mm); Δ , variable substrate: PA, Mg^{2+} ions (5 mm), PA ($10^{-3}M$); $\bullet$, variable substrate: ADP (Mg^{2+} ions also varied to give Mg :ADP=5:1). Total volume 1.0 ml.; temp. 5°. $v = \mu$ moles/mg./min.

calculated that the K_m value of APK for PA was $1.2 \times 10^{-3} \text{ M}$. The same value was obtained when the Mg^{2+} ion concentration was increased to 10^{-2} M . It will be noted that identical maximum velocities were obtained irrespective of whether ADP or PA was the variable substrate. Thus in each case the concentrations of the non-variable substrates were sufficient to saturate the enzyme.

(b) Reverse reaction. The relationship between the initial reaction velocity of the reverse reaction and the ATP concentration was determined under conditions where the Mg^{2+} ion concentration was fixed at 10^{-2} M and also where the ratio of Mg^{2+} ion:ATP was maintained at 1:1. The results plotted according to the method of Lineweaver & Burk (1934) are shown in Fig. 6. In the latter case the reaction velocity at each level of ATP was lower than that obtained when the Mg^{2+} ion concentration was fixed at 10^{-2} M except for the one instance where the Mg^{2+} ion:ATP ratio of 1:1 is common to each plot.

It can be seen (Fig. 6) that when the arginine concentration was varied and the other components of the system were held constant a straight line was obtained when the results were plotted as described by Lineweaver & Burk (1934). It will be noted that the maximum velocity was greater than that obtained with ATP as the variable substrate. It would seem therefore that the enzyme was not saturated with respect to arginine under the conditions chosen for the determination of the K_m of

APK for ATP. The K_m of APK for arginine was calculated to be $3.2 \times 10^{-3} \text{ M}$.

A summary of the K_m and $V_{\max}$ values obtained for the substrates of APK is given in Table 2.

Inhibition of the forward and reverse reactions by the reaction products. It was of interest to determine whether or not the products of the forward and the reverse reactions had any effect on the velocity of the reaction. For technical reasons it was not possible to test the effect of arginine in the forward reaction or PA in the reverse reaction. However, it was possible to test the effect of ATP in the forward reaction and ADP in the reverse reaction. Table 3 shows that ATP does not inhibit the forward reaction when added at the same concentration as the ADP, and that it is only when the ATP concentration is 10 times that of the ADP that inhibition occurs. There was only a low degree of inhibition by adenylic acid. These results suggest that the affinity of APK for ADP under the conditions of the forward reaction is very much greater than for ATP or adenylic acid. Alternatively, the results could be explained as being due to a reduction of the Mg^{2+} ion concentration and hence the concentration of the active Mg -APK complex as a result of the formation of Mg -ATP and Mg -adenylic acid complexes.

In contrast to the above results there is marked inhibition of the reverse reaction by ADP. Fig. 7 shows that the inhibition is competitive, the degree of inhibition depending both on the concentration of

Fig. 6. Effect of arginine and ATP concentrations on the initial velocity of the reverse reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained *N*-ethylmorpholine (0.06 M , pH 8.4) and APK ($15 \mu\text{g. of protein/ml.}$). ●, Variable substrate: arginine, Mg^{2+} ions (10^{-2} M), ATP (10^{-3} M); ○, variable substrate: ATP, Mg (10^{-2} M), arginine (10^{-2} M); Δ, variable substrate: ATP (Mg^{2+} ions also varied to give $\text{Mg}:\text{ATP} = 1:1$). Total volume 1.0 ml. ; temp. 5° . $v = \mu\text{moles/mg./min.}$

Table 2. K_m values and maximum velocities

Conditions were as described in Figs. 5 and 6.

Substrate	K_m (mm)	$V_{\max}$ ($\mu\text{moles/mg./min.}$)	
Forward reaction (pH 7.2)			
PA	1.2	61.0	—
ADP	2.4	61.0	Mg^{2+} ions constant
	3.0	56.0	Mg^{2+} ions: ADP = 5:1
Reverse reaction (pH 8.4)			
Arginine	3.2	25.0	—
ATP	2.5	10.5	Mg^{2+} ions constant
	3.8	9.8	Mg^{2+} ions: ATP = 1:1

Table 3. Inhibition of the forward reaction by ATP and AMP

The reaction mixture contained PA ($5 \times 10^{-3} \text{ M}$), ADP (10^{-3} M), Mg^{2+} ions (10^{-2} M), *N*-ethylmorpholine (0.06 M , pH 7.2) and APK ($1 \mu\text{g. of protein/ml.}$). Total volume, 1.0 ml. Temp. 5° .

Addition	Concn. (mm)	Inhibition (%)
ATP	10	54
	1	0
Adenylic acid	4	26
	1	16

the ADP and ATP. Calculation of the K_i value for ADP gave a figure of $1.5 \times 10^{-3} \text{ M}$, which is the same as that obtained for the dissociation constant of the APK-ATP complex.

Effect of temperature on the reaction velocity. The effect of temperature on the reaction velocity is shown in Fig. 8. Two Arrhenius plots are shown; one for the forward reaction at pH 7.2 and the other for the reverse reaction at pH 8.4. In order to measure true initial reaction velocities, the same enzyme concentration was used at all temperatures, but the reaction time was progressively decreased with increasing temperature. The forward reaction followed

Fig. 7. Competitive inhibition of the reverse reaction by ADP with ATP as the variable substrate. The results are plotted according to the method of Lineweaver & Burk (1934). The reaction mixture contained *N*-ethylmorpholine (0.06 M, pH 8.4), arginine (10^{-2} M), Mg^{2+} ions (10^{-2} M) and APK (15 μg . of protein/ml.). Total volume, 1.0 ml.; temp., 5°. ○, No added ADP; ●, ADP (2 mM); Δ, ADP (2 mM). $v = \mu\text{moles/mg.}/\text{min}$.

Fig. 8. Effect of temperature on the initial velocity of the forward and reverse reaction. The results are graphed as Arrhenius plots. Conditions for the forward reaction were as described in Fig. 2; conditions for the reverse reaction were as described in Fig. 1. $T = \text{absolute temp.}$; $k = \mu\text{moles/mg.}/\text{min}$. ○, Forward reaction; Δ, reverse reaction.

the Arrhenius equation over the range 0–35°. From the slope of the line, the apparent activation energy was calculated to be 9500 cal./mole. The Arrhenius equation held also for the reverse reaction over the same temperature range, although there was a sharp break at about 10°. The apparent activation energy was 21 500 below 10° and 9800 cal./mole above 10°.

Equilibrium studies. The effect of pH and Mg^{2+} ion concentration on the percentage phosphorylation of arginine is shown in Fig. 9. It is apparent that increasing pH favours the phosphorylation of arginine at the three concentrations of Mg^{2+} ions used and that the degree of phosphorylation is dependent on the Mg^{2+} ion concentration at any particular pH. As the Mg^{2+} ion concentration decreases the percentage phosphorylation of arginine increases.

Enzymic activity in the absence of added Mg^{2+} ions. A comparison was made of the K_m values of APK for ADP and PA in the presence and absence of Mg^{2+} ions, and for this purpose an APK preparation before dilution with glycerol was dialysed against 0.01 M phosphate buffer (pH 7.4). In the absence of Mg^{2+} ions the time-activity curve (with a protein concentration of 5 $\mu\text{g}/\text{ml.}$) showed a rapid fall off of the reaction rate. This occurred after 4 min. when only 0.13 μmole of arginine had been released in the presence of 5 μmoles of PA and 1 μmole of ADP; up to 4 min. the reaction was linear. Thus for the determination of the K_m values the volume of the reaction mixture was doubled and the reaction time reduced to 3 min.

Fig. 9. Effect of pH and Mg^{2+} ion concentration on the percentage phosphorylation of arginine. The reaction mixture contained PA (5 mM), ADP (2 mM), *N*-ethylmorpholine (0.06 M) and APK (46 μg . of protein/ml.); incubated for 2 hr. at 5°. The pH was checked by means of a glass electrode in a duplicate set of tubes. ○, Mg^{2+} ions (10^{-2} M); Δ, Mg^{2+} ions (2 mM); ●, Mg^{2+} ions ($2 \times 10^{-4} \text{ M}$).

It was found that the K_m of APK for ADP was $1.5 \times 10^{-4} \text{ M}$ as compared with $3.0 \times 10^{-4} \text{ M}$ in the presence of Mg^{2+} ions, whereas the K_m of APK for PA was $2.2 \times 10^{-3} \text{ M}$ as compared with $1.2 \times 10^{-3} \text{ M}$ in the presence of Mg^{2+} ions.

The dialysed APK preparation also catalysed the reverse reaction in the absence of Mg^{2+} ions, but the amount of protein required to obtain suitable reaction rates was so high that it interfered with the P estimation. Hence it was not possible to determine the K_m values of APK for ATP and arginine.

Inhibition of the forward reaction by Ca^{2+} ions. When Ca^{2+} ions were added to the forward reaction catalysed by APK in the absence of Mg^{2+} ions, there was no inhibition up to concentrations of 10^{-2} M . On the other hand, in the presence of 10^{-2} M Mg^{2+} ions, 10^{-2} M Ca^{2+} ions produced an inhibition of 72%. Thus the effect of Ca^{2+} ions is to lower the increase in activity due to Mg^{2+} ions. Further investigation of the inhibition of the reaction by Ca^{2+} ions showed that it was competitive (Fig. 10), the degree of inhibition depending upon the relative concentrations of Mg^{2+} and Ca^{2+} ions. It was calculated that the K_i value for Ca^{2+} ions was $8.3 \times 10^{-5} \text{ M}$ as compared with a dissociation constant of $3.6 \times 10^{-4} \text{ M}$ for Mg -APK. The affinity of APK for Ca^{2+} ions is therefore much greater than its affinity for Mg^{2+} ions.

DISCUSSION

The nature of the activation produced by Mg^{2+} ions in transphosphorylation reactions is of some interest; on general grounds it is possible that a

Mg -ATP complex is formed which acts as the 'active' substrate. Such a conclusion has been reached by Kuby, Noda & Lardy (1954), Hers (1952) and Liébecq (1953) as a result of the study of reactions catalysed by creatine phosphokinase, fructokinase and hexokinase respectively. They found that maximum reaction velocities were attained when the Mg^{2+} ion and ATP concentrations were approximately equal. A similar result has also been obtained in the present work but seems open to other interpretations, for Mg^{2+} ions could also act by influencing the velocity of the breakdown of the enzyme-substrate complex, or by reacting with the apoenzyme to form an active Mg -enzyme complex.

In assessing the validity of the conclusion drawn by the above workers it should be remembered that it is based upon a relationship obtained when enzymic activities, at varying concentrations of ATP, are plotted against the concentration of added Mg^{2+} ions, and that this is tacitly assumed to be equal to the concentration of free Mg^{2+} ions. Such an assumption is, however, invalid, for as Burton & Krebs (1953) have shown, both ATP and ADP form complexes with Mg^{2+} ions, so that the concentration of 'free Mg^{2+} ions', 'free ATP' and Mg -ATP will vary according to the amounts of Mg^{2+} ions and ATP added.

The relationship between the velocity of the APK reaction (in both the forward and reverse directions) and the Mg^{2+} ion concentration is consistent with the idea that Mg^{2+} ions react with the enzyme in the ratio of one Mg^{2+} ion to each active centre, according to the equation

Thus the Mg -APK complex can be considered as the active-enzyme complex. The dissociation constant of Mg -APK as determined from the plot of $1/v$ against $1/\text{Mg}^{2+}$ ions (where the concentration of Mg^{2+} ions is taken to be the same as that added) will not be a true dissociation constant for the Mg -APK complex. The value so calculated will lie between the true dissociation constant of the Mg -APK complex and that of either Mg -ATP or Mg -ADP, and will depend on the relative dissociation constants of Mg -APK and Mg -ATP or Mg -ADP. It would be expected that, under the same experimental conditions and provided that there are no secondary reactions between Mg^{2+} ions and the substrates, the dissociation constant of the Mg -enzyme complex would be the same irrespective of whether it was determined as a result of study of the forward or reverse reactions. This expectation is not realized experimentally, for the results (Table 1) show that at pH 7.9 the value is lower when calculated from the forward reaction than when calculated from the reverse. Thus less Mg^{2+} ions are apparently required to half-saturate

Fig. 10. Competitive inhibition of the forward reaction by Ca^{2+} ions when Mg^{2+} ion concentration was varied. The reaction mixture contained PA (5 mM), ADP (mM), *N*-ethylmorpholine (0.06 M, pH 7.2) and APK (1.5 μg . of protein/ml.). Total volume, 1.0 ml., temp. 5°. ○, No added Ca^{2+} ions; ●, Ca^{2+} ions ($2 \times 10^{-4} \text{ M}$); Δ, Ca^{2+} ions ($5 \times 10^{-4} \text{ M}$).

the enzyme in the presence of ADP than in the presence of ATP. Such a result is, however, consistent with the interpretation that Mg^{2+} ions react with the enzyme, for the dissociation constant of Mg-ADP is greater than that of Mg-ATP and thus more Mg^{2+} ions would be available to interact with the enzyme. It can also be seen (Table 1) that a fivefold increase in ADP concentration results in an increase in the dissociation constant of Mg-APK, which is consistent with the idea that ADP reduces the amount of Mg^{2+} ions available for reaction with the enzyme.

The differences in the values for the dissociation constant may be accounted for by the fact that the abscissae values of the plots (Figs. 3, 4) represent the concentrations of Mg^{2+} ions added and thus do not allow for any reduction as a result of interaction with ADP or ATP. It is, however, possible to calculate the 'free Mg^{2+} ion' concentration from the dissociation constants of Mg-ATP and Mg-ADP (Burton & Krebs, 1953), and for the purpose of calculation it has been assumed that Mg^{2+} ions react, in the systems investigated, only with ATP, ADP and APK; all other possible reactions have been neglected. On this basis the Mg^{2+} ions which are not in combination with ATP or ADP are referred to as 'free Mg^{2+} ions'. If the reciprocals of the values so obtained are plotted against $1/v$ the dissociation constants of Mg-APK as determined under various conditions may be calculated and are shown in bold type in Table 4. It will be seen that these values are very close to each other and differ much less than those calculated directly from Figs. 3 and 4. They are also considerably lower than these latter, which are dependent upon the concentration of ATP or ADP in the system and the dissociation constants of Mg-ATP and Mg-ADP. These results indicate that the role of Mg^{2+} ions in the APK reaction lies in a combination of the metal with the enzyme and that the formation of Mg-ADP and Mg-ATP are inevitable side reactions.

A characteristic feature of the results plotted in Fig. 1 is that maximum enzymic activities are obtained at varying concentrations of Mg^{2+} ions,

depending on the concentrations of ATP. If, however, the activities are replotted against the concentrations of 'free Mg^{2+} ions' determined as before, it can be shown that maximum enzymic activity is obtained when the latter is $2 \times 10^{-3} M$ and that this value is independent of the concentration of ATP. It is also worthy of note that no relationship between enzymic activity and Mg-ATP concentration could be demonstrated. In the forward reaction (Fig. 2) maximum enzymic activity is again reached at Mg^{2+} ion concentrations which are dependent upon the ADP concentration. There is, however, no simple relationship between the concentrations of ADP and Mg^{2+} ions when maximum enzymic activity is reached. If these results are recalculated and the enzymic activity is plotted against 'free Mg^{2+} ion' concentration, it is found that maximum enzymic activity is reached at each level of ADP when the concentration of 'free Mg^{2+} ions' is $2.5 \times 10^{-3} M$. This value is similar to that obtained for the reverse reaction.

It will be noted that the maximum velocities in the forward reaction (Fig. 2) fall within a much narrower range of Mg^{2+} ion concentrations, and that they are much closer to the true values than is the case with the reverse reaction (Fig. 1). These findings are due to the fact that the amounts of ADP added in the forward reaction were less than the amounts of ATP added for the reverse reaction, and since the dissociation constant of Mg-ADP is higher than that of Mg-ATP it follows that the values for concentrations of 'free Mg^{2+} ions' in the forward reaction must be closer to those for total Mg^{2+} ions than is the case in the reverse reaction.

The decrease in velocity of the forward reaction (Fig. 2) in the presence of high concentrations of Mg^{2+} ions is due to a reduction of 'free ADP' concentrations, as a result of interaction with Mg^{2+} ions. In this case the ratio of Mg^{2+} ions:ADP was much greater than was the ratio of Mg^{2+} ions:ATP in the reverse reaction, where no fall off of reaction velocity was noted (Fig. 1).

When the K_m of APK for ADP was determined in the presence of a constant amount of Mg^{2+} ions

Table 4. Dissociation constants of Mg-APK from plots of $1/v$ against $1/\text{total Mg}$ and $1/\text{free } Mg^{2+}$ ions*

Determined in the presence of	Concn. of ADP or ATP (mm)	Calculated dissociation constant		
		pH 7.2	pH 7.9	pH 8.4
ADP	1	$5.4 \times 10^{-4*}$ $3.6 \times 10^{-4†}$	4.3×10^{-4} 3.1×10^{-4}	— —
	5	—	1.0×10^{-3} 3.3×10^{-4}	— —
ATP	5	—	8.7×10^{-4} 2.4×10^{-4}	1.5×10^{-3} 4.7×10^{-4}

* Calculated from a plot of $1/v$ against $1/\text{total } Mg^{2+}$ ions.

† Calculated from a plot of $1/v$ against $1/\text{free } Mg^{2+}$ ions*.

a Michaelis-Menten relation was obtained only over a limited range of ADP concentrations, for the velocity was much less at lower levels of ADP than that required. This was due to a reduction of the ADP concentrations by Mg^{2+} ions, for a Michaelis-Menten relation was obtained over the entire range of ADP concentrations when $1/v$ was plotted against $1/\text{'free ADP'}$ rather than $1/\text{total ADP}$. The K_m value obtained from this plot (Table 5) must be regarded as the true value and it will be noted that it is appreciably lower than that calculated on the basis of total ADP. When the Mg^{2+} ion concentration was varied simultaneously with the ADP to maintain a Mg:ADP ratio of 5:1 a Michaelis-Menten relation was obtained over the range of ADP concentrations studied. The K_m value (Table 5) was close to that obtained when the Mg^{2+} ion concentration was kept constant, both values being calculated from a plot of $1/v$ against $1/\text{total ADP}$. However, the K_m value calculated from a plot of $1/v$ against $1/\text{'free ADP'}$ is considerably higher than what is considered as the true value, and higher than that obtained on the basis of total ADP. This high value is probably due to the fact that as the Mg^{2+} ion concentration was reduced concomitantly with the ADP concentration (maintaining a Mg:ADP ratio = 5:1) it limited the concentration of the Mg-APK complex. Thus the fall off in rate was due to a reduction of both the ADP and Mg-APK concentrations.

When the ATP concentration was varied in the presence of a fixed concentration of Mg^{2+} ions and when both the ATP and Mg^{2+} ion concentrations were varied so as to maintain a Mg:ATP ratio of 1:1, a Michaelis-Menten relation between the reaction velocity and the ATP concentration was found (Fig. 6). It can be seen (Table 5) that although the K_m values calculated from a plot of $1/v$ against $1/\text{total ATP}$ differ slightly, they are considerably different from those calculated from a plot of $1/v$ against $1/\text{'free ATP'}$. The value obtained from the latter plot in the presence of a fixed amount of Mg^{2+} ions must be considered as the true K_m value of APK for ATP. The high value calculated from a similar plot of the results obtained when the Mg^{2+}

ion concentration was varied is probably due to the concentration of Mg^{2+} ions limiting the concentration of Mg-APK as was the case with the forward reaction.

The influence of Mg^{2+} ions on the reaction can also be seen from the equilibrium studies. As the concentration of Mg^{2+} ions is increased, the equilibrium shifts in favour of arginine and ATP. This can be explained as being due to the greater affinity of Mg^{2+} ions for ATP than for ADP. If Mg-ATP were the true substrate for the reaction, it would be expected that the equilibrium would be displaced in favour of PA and ADP as the concentration of Mg^{2+} ions, and thus of Mg-ATP, was increased.

There is additional supporting evidence to suggest that the function of Mg^{2+} ions is to form a complex with the apoenzyme. In the experiments carried out with the dialysed APK preparation to which no Mg^{2+} ions were added the enzyme showed activity in both the forward and reverse reactions. It was found that the K_m of APK for ADP was lower than when Mg^{2+} ions were present although higher than that calculated on the basis of the 'free ADP' present when Mg^{2+} ions were added; on the other hand, the K_m value of APK for PA was increased. Although no explanation can be offered for the higher value, the results do indicate that the binding of Mg^{2+} ions by PA must be sufficiently weak so as to have no effect on the reaction. The activity of the enzyme in the absence of added Mg^{2+} ions is probably due to Mg^{2+} ions bound to the enzyme. Spectrographic analysis (Morrison *et al.* 1957) has shown that Mg^{2+} ions are present in a dialysed APK preparation.

Further evidence for the idea that Mg^{2+} ions react with APK to form an active Mg-APK complex comes from the results of the inhibition of APK by Ca^{2+} ions. Ca^{2+} ions have no effect on the forward reaction catalysed by APK in the absence of Mg^{2+} ions, whereas they competitively inhibit the enzyme in the presence of Mg^{2+} ions. Thus the interaction of Ca^{2+} ions and ADP to form Ca-ADP does not appreciably reduce the concentration of free ADP. This can be explained by the fact that the K_m value of APK for ADP ($4.3 \times 10^{-5} M$) is much lower than the dissociation constant of Ca-ADP ($1.8 \times 10^{-4} M$; Distefano & Neuman, 1953). The inhibition of the reaction when Mg^{2+} ions are present must therefore be due to the competition by Ca^{2+} and Mg^{2+} ions for the same site on the enzyme surface. It is noteworthy that the affinity of APK for Ca^{2+} ions is greater than for Mg^{2+} ions, although there is no activation by Ca^{2+} ions. These results also imply that in APK as isolated, the linkage of Mg^{2+} ions to the enzyme is different from and stronger than that resulting from the addition of Mg^{2+} ions to the enzyme system.

Table 5. K_m values of APK for ADP and ATP

Substrate	Concn. of Mg^{2+} ions (M)	K_m value (M)	
		(a)*	(b)†
ADP	5×10^{-3}	2.4×10^{-4}	4.3×10^{-5}
	Mg:ADP = 5:1	3.0×10^{-4}	6.6×10^{-4}
ATP	1×10^{-2}	2.5×10^{-3}	2.8×10^{-4}
	Mg:ATP = 1:1	3.8×10^{-3}	6.3×10^{-3}

* (a) Calculated from a plot of $1/v$ against $1/\text{total substrate}$.

† (b) Calculated from a plot of $1/v$ against $1/\text{'free substrate'}$.

The competitive inhibition of the reverse reaction by ADP and the inhibition of the forward reaction by ATP suggest that ATP and ADP are bound to APK at the same site on the enzyme surface. It is possible that this linkage is through Mg^{2+} ions, as this metal complexes predominantly in *O*-type linkages (Williams, 1953). The fact that ATP cannot be replaced by inosine triphosphate in the reaction (Morrison *et al.* 1957) indicates that the adenine moiety of ATP is also involved in the linkage of ATP or ADP to the enzyme.

It is believed that the present results indicate that Mg^{2+} ions react with the apoenzyme to form an active Mg -APK complex, and that reaction of free ADP or ATP with the active enzyme involves linkage with Mg^{2+} ions. It is possible that PA and arginine are attached at another site on the enzyme surface in sufficiently close proximity to the ADP-ATP site so that P can be directly transferred without liberation as inorganic P. This hypothesis, inasmuch as it concerns the reaction investigated, may be schematically represented as in Fig. 11.

Although this scheme is based on the results of experiments in which Mg^{2+} ions were used to activate the enzyme, it should be borne in mind that APK is also activated by Mn^{2+} ions. It is, therefore, probable that the mechanism of the activation of APK by Mn^{2+} ions is similar to that postulated for Mg^{2+} ions.

Fig. 11. Schematic representation of the mechanism of the transphosphorylation reaction catalysed by APK. All reactions are reversible. The abbreviations AP and A represent phosphoarginine and arginine respectively.

It should be pointed out that recalculation of the results of Kuby *et al.* (1954), Hers (1952) and Liébecq (1953) might well indicate that a similar central reaction, i.e. formation of a metal-enzyme complex, is concerned in the reactions catalysed by creatine phosphokinase, fructokinase and hexokinase, and that this type of reaction may be one generally applicable to phosphate-transfer reactions.

SUMMARY

1. The kinetics of the reaction, phosphoarginine + adenosine diphosphate $\rightleftharpoons$ arginine + adenosine triphosphate, catalysed by arginine phosphokinase have been studied.

2. It has been shown that when enzymic activity is plotted against the total Mg^{2+} ion concentration, maximum activity is reached at a concentration of Mg^{2+} ions which is dependent on the concentration of adenine diphosphate (ADP) or adenosine triphosphate (ATP). When enzymic activity is plotted against the concentration of 'free Mg^{2+} ions', maximum activity is reached at a Mg^{2+} ion concentration which is independent of the concentration of ADP or ATP.

3. A Michaelis-Menten relation between enzymic activity and the concentration of Mg^{2+} and Mn^{2+} ions was demonstrated. The dissociation constants of the magnesium-arginine phosphokinase complex, determined under various conditions, were calculated from plots of $1/v$ against $1/\text{total } Mg^{2+}$ ions and $1/v$ against $1/\text{'free } Mg^{2+}$ ions'.

4. The K_m values of the four substrates of the reaction were determined. Those for ADP and ATP were calculated from plots of $1/v$ against $1/\text{total substrate}$ and $1/v$ against $1/\text{'free substrate'}$.

5. The forward reaction was inhibited by ATP and adenylic acid; the reverse reaction was inhibited competitively by ADP. It was concluded that ADP and ATP are bound to the enzyme at the same site.

6. There was competitive inhibition of the forward reaction by Ca^{2+} ions.

7. The effect of temperature on the velocity of the forward and reverse reactions was studied and the apparent activation energies were calculated.

8. The equilibrium of the reaction was influenced by pH and the concentration of Mg^{2+} ions.

9. The conclusion was reached that Mg^{2+} ions react with arginine phosphokinase to form an active magnesium-arginine phosphokinase complex which in turn reacts with the free forms of ATP and ADP.

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Studies in the Biochemistry of Micro-organisms

99. METABOLIC PRODUCTS OF *ASPERGILLUS VERSICOLOR* (VUILLEMIN) TIRABOSCHI*

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The first examination in this Laboratory of the metabolic products obtained from the mycelium of *Aspergillus versicolor* (Vuillemin) Tiraboschi was undertaken by Abou-Zeid (1953). He isolated in very small yield two new coloured crystalline metabolites, *A*, m.p. 240–241°, and *B*, m.p. 233–234°, in addition to mannitol. The amounts of *A* and *B* obtained were insufficient for complete characterization but a few properties and colour reactions were noted.

One of us (Hammady, 1954), using a later isolation of the same organism, obtained the same three products. Product *A*, formed in relatively good yield, was obtained as pale-yellow needles of m.p. 247°, and was allocated the molecular formula $C_{18}H_{18}O_6$. The general properties, derivatives and some breakdown products were described.

Davies, Roberts & Wallwork (1956) recently announced that early in 1955 they had commenced an investigation of *A. versicolor* and had obtained from the dried mycelium of one strain a metabolite crystallizing in pale-yellow needles of m.p. 243°. They drew attention to the work of Hatsuda & Kuyama (1954) and of Hatsuda, Kuyama & Terashima (1954), who had isolated the same metabolite and named it sterigmatocystin. Hatsuda *et al.* (1954) ascribed to sterigmatocystin the molecular formula $C_{15}H_{12}O_6$ and allocated to it a structural formula. Davies *et al.* (1956), adopting the name sterigmatocystin, determined the molecular weight by the Rast method and by the X-ray crystallographic method. From these results and the combustion figures they concluded that the molecular formula for sterigmatocystin is $C_{18}H_{14}O_6$,

both empirical and structural formulae given by Hatsuda *et al.* (1954) being thus incorrect.

It was then evident that product *A* isolated in this Laboratory was the same as sterigmatocystin. Through the courtesy of Dr Roberts we were able to compare our product directly with a specimen isolated by the Nottingham School; a mixed-melting-point determination showed they were identical. Since the work carried out on this subject in this School has been reported only in Ph.D. theses of London University it is appropriate to record briefly for wider circulation the results therein embodied which relate to the metabolic products of *A. versicolor*.

Some strains of the fungus when grown on Czapek–Dox medium produced a coloured mycelium, which, after drying, was extracted with solvents. Light petroleum afforded an orange-coloured solid from which product *A* (sterigmatocystin) was obtained. On subsequent ether extraction of the mycelium product *B* was isolated. It formed orange needles of m.p. 233–234°. Further extraction of the mycelium with methanol afforded mannitol.

Sterigmatocystin is optically active and contains one methoxyl group. The following derivatives have been prepared: diacetyl, monomethoxy, monomethoxy compounds and a brominated derivative. When refluxed with 25% ethanolic potassium hydroxide, sterigmatocystin furnished an optically inactive isomer which afforded a monomethyl ether and a diacetate. This product, which we term *iso*-sterigmatocystin, shows an ultraviolet-absorption curve very similar to that of sterigmatocystin, indicating that the conjugated system giving rise to the absorption is still present. Some change other than mere racemization must have occurred,

* Part 98: Neelakantan, Pocker & Raistrick (1956).