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Kinetic Studies on Rabbit-Muscle Myokinase

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Myokinase (adenylate kinase) catalyses the reaction:

Highly purified preparations of this enzyme have been obtained from rabbit muscle by Noda & Kuby (1956, 1957*a, b*) and Callaghan (1956, 1957*a, b*), and the preparation of the last-named author, which appears to contain a single protein component, has been used for the present studies of the mechanism of myokinase action. Several possible mechanisms by which myokinase might catalyse transphosphorylation among adenosine nucleotides were considered by Whittam, Bartley & Weber (1955). The enzyme may accept the terminal phosphate of adenosine triphosphate (ATP) or adenosine diphosphate (ADP), thus forming an intermediate phosphorylated protein by the reactions:

or may act by forming an enzyme-nucleotide complex according to the following scheme:

A further possibility is expressed by the reactions:

By use of the purified enzyme, the hypothetical phosphorylated enzyme intermediate was tested for directly by incubation with ATP of comparable molar concentration in the region of the Michaelis constant (K_m) value for the substrate; in most experiments of this kind, the ATP was labelled with ^{32}P in the β - and γ -phosphate groups. Kinetic studies were also undertaken to determine the affinity of the enzyme for its substrates, and a special study of the reverse reaction was made to

determine whether the enzyme contained sites to activate two molecules of substrate simultaneously or a single site (as expressed by equations 3 and 4 respectively). The preparation was also tested for other transphosphorylating enzymes.

EXPERIMENTAL

Adenosine triphosphate. This was obtained as the crystalline disodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A. Chromatographic analysis of this material by the method of Krebs & Hems (1953) indicated that 94% of the total phosphate was present in the form of ATP, 4–5% as ADP and approximately 2% as inorganic orthophosphate (PI). These figures are discussed in greater detail below.

Adenosine diphosphate. This was obtained as the crystalline disodium salt from the Sigma Chemical Co. and was found on analysis to contain less than 2% of AMP.

Adenosine monophosphate. This was obtained from Roche Products Ltd., Welwyn Garden City, Herts. Solutions of AMP were brought to pH 7.5 before use.

Inosine and uridine phosphates. The authors gratefully acknowledge the gift of small amounts of these compounds by Dr N. L. Blumsom; all were 5'-phosphate esters. The inosine 5'-phosphate preparation contained much inosine 5'-diphosphate (see below) but was not purified further.

Cytidylic acid and guanylic acid. These were obtained as the 3'-phosphate esters from L. Light and Co. Ltd., Slough, Bucks.

Adenosine 5'-monosulphate. A small amount of this substance was the generous gift of Professor Kunio Yagi.

$\text{KH}_2^{32}\text{PO}_4$. This was supplied by The Radiochemical Centre, Amersham, Bucks.

Chromatographically pure adenosine triphosphate. ATP (Sigma Chemical Co.) was rechromatographed with the intention of removing traces of ADP. The dissolved substance was applied as a streak to Whatman no. 3 paper and the procedure of Krebs & Hems (1953) was followed. The ATP on the paper was localized by illumination with the 2537 Å Hg line. This region was cut out, washed with ether to remove traces of solvents and eluted by downward chromatography with water. Chromatographic analysis of this product is described below.

[^{32}P]Adenosine triphosphate. This compound, containing equal specific activity in the β - and γ -phosphate groups ($\text{AT}\beta\gamma^{32}\text{P}$) was prepared by a procedure (similar to that of Hems & Bartley, 1953) of Dr M. L. Bobb (personal communication). The gift of an initial sample of $\text{AT}\beta\gamma^{32}\text{P}$ from Dr Bobb is gratefully acknowledged. A low-phosphorus yeast extract was prepared according to Eggleston (1954). A portion (2 ml.) of the extract was shaken aerobically at room temperature with 0.1 ml. of 0.02 M-ATP (adjusted to pH 6.8) and 2 ml. (5 mc) of $\text{KH}_2^{32}\text{PO}_4$ solution (pH 7.0) for 4 hr. The mixture was transferred to a conical centrifuge tube and deproteinized with 0.5 ml. of 50% (w/v) trichloroacetic acid soln. The supernatant obtained on centrifuging was cooled to 0° and applied in a streak to Whatman no. 3 paper, the $\text{AT}\beta\gamma^{32}\text{P}$ being separated by chromatography as described above. A representative preparation of 0.25 μ -mole of $\text{AT}\beta\gamma^{32}\text{P}$ gave a total count of 2.9×10^6 /min. in solution. Of the total counts, ATP contained 74%, ADP 5%, AMP 0.2% and PI 20%. The specific activity of the

β - or γ -phosphate groups of ATP was 2.1×10^6 counts/min./ μ g. of phosphorus, the α -phosphorus atom being negligibly labelled. The preparation was stored on a Whatman no. 3 paper strip at -15° , and small quantities were eluted with ATP solution for use in experiments.

Myokinase. This was prepared by the method of Callaghan (1957b) and was dialysed in collodion tubes coated with cytochrome *c* (Callaghan, 1957b).

Assay methods

Myokinase. For the routine assay of myokinase preparations the method of Oliver (1955) was used. In kinetic studies, the initial velocity of myokinase activity was measured by chromatographic assay as previously described (Callaghan, 1957b). It was found that the chromatographic procedure of Krebs & Hems (1953) was also suitable for the separation of uridine di- and mono-phosphate from the adenosine nucleotides, the former compounds having similar R_f values to inosine di- and mono-phosphate respectively. Cytidylic acid had an almost identical R_f value with that of AMP.

Radioactivity measurements. These were made in an M6 liquid counter (Veall, 1948), which was periodically cleaned with a dilute solution of detergent (Teepol; Shell) to remove adsorbed protein and traces of radioactive material. Areas on chromatograms were cut as circles from the paper and counted in an end-window counter. Corrections were made for dead-time and background counts. No correction was made for isotope decay, as standard solutions of $\text{AT}\beta\gamma^{32}\text{P}$ were counted with all specimens. Spectrophotometric measurements were made with an SP. 500 Unicam spectrophotometer.

Estimation of nucleotides in solutions of protein. In some experiments it was necessary to estimate the sum of the concentrations of ATP, ADP and AMP in solution by spectrophotometry (these compounds have identical absorption spectra in the ultraviolet region). It was also necessary to correct for the light absorption due to myokinase protein present. As will be shown below, the characteristic absorption of ultraviolet light by the enzyme and the nucleotides in solution is purely additive. The following equations were therefore applicable.

Let the ratio of light extinction by myokinase protein at the wavelengths

$$260 \text{ m}\mu \text{ and } 280 \text{ m}\mu \text{ be } \frac{P_{260}}{P_{280}} = \pi.$$

Similarly, for ATP, ADP and AMP, the ratio of extinction at the same wavelengths

$$\frac{N_{280}}{N_{260}} = \nu.$$

(At the low concentrations used for measurement, where the total extinction was 0.1–0.2, the average values for π and ν were 1.28 and 0.178 respectively.)

The total absorption of light at 260 m μ , $E_{260} = P_{260} + N_{260}$.

Similarly, the total absorption at 280 m μ ,

$$E_{280} = P_{280} + N_{280}.$$

$$\text{Therefore } E_{280} = \pi P_{260} + \nu N_{260} \quad (5)$$

$$\text{and } P_{260} = E_{280} - N_{280}. \quad (6)$$

Combining equations (5) and (6),

$$E_{280} = \pi E_{260} + (\nu - \pi) N_{260}.$$

$$\text{Therefore } N_{260} = \frac{E_{280} - \pi E_{260}}{(\nu - \pi)}. \quad (7)$$

From equation (7) it is possible to obtain the extinction at 260 m μ due to nucleotide alone with reasonable precision, since $(\nu - \pi)$ is approximately unity.

RESULTS

Tests for a phosphorylated enzyme intermediate

Direct method. To determine whether myokinase formed a phosphorylated intermediate, according to equation (2), ATP and myokinase were incubated together in comparable molar concentrations which were of the order of the K_m concentration of ATP. The results of this experiment are given in Table 1, and show that there was no alteration in the proportions of ATP and ADP; this indicates that myokinase did not form a phosphorylated intermediate, otherwise the enzyme (0.9 μ mole) would have been approximately half-saturated with phosphate, with the concomitant dephosphorylation of about 26 % of the ATP present to form ADP. Table 1 also shows that the chromatographically separated specimen of ATP had the same proportions of ATP, ADP and PI as the original preparation from which it was made. The conclusion is drawn that some hydrolysis of ATP (about 5 %) occurs during chromatography in the first solvent of Krebs and Hems (*isopropyl ether-formic acid*), and that the Sigma Chemical Co. preparation

Table 1. *Test for phosphorylated enzyme intermediate*

In a total volume of 4 ml. the following constituents were incubated at 20° and pH 7.4: 18.9 mg. of pure myokinase (0.865–0.9 μ mole); 0.01 ml. of m-MgCl₂; 20 mg. of cysteine hydrochloride (neutralized to pH 7.4); 1.7 μ moles of chromatographed ATP (final concentration 0.426 mM; K_m of ATP = 0.3 mM, see below). After 1 min. 1 ml. of 30 % (w/v) trichloroacetic acid was added, and the mixture was cooled to 0° and centrifuged. A portion (172 μ l.) of the supernatant was applied in a streak on Whatman no. 1 paper and chromatographed according to Krebs & Hems (1953). 93 % of the total phosphates added to the above reaction mixture was recovered from the trichloroacetic acid supernatant.

Area	Percentage of total phosphorus as phosphate in chromatographic spots		
	ATP (Sigma Chemical Co.)	Chromatographically separated ATP	Trichloroacetic acid supernatant from enzyme
ATP	94.5	93.5	95.0
ADP	4.5	4.5	4.0
PI	1.0	2.0	1.0

of ATP contains a higher percentage of this compound than this analysis would indicate, being possibly 98–99 % pure as claimed by the manufacturers.

Experiments with radioactive adenosine triphosphate. The possible phosphorylation of myokinase by ATP was also tested for by incubation of the enzyme with AT β ³²P. Attempts were made to separate the enzyme-³²PO₄ complex from the added nucleotide by means of prolonged dialysis and zone electrophoresis.

It was expected that, if such a complex were formed, its partial or total separation from the radioactive nucleotide would be reflected in a high specific radioactivity of the enzyme, for which any residual nucleotide could not be responsible, i.e. the ratio $\frac{\text{counts/unit time}}{\text{moles of adenosine nucleotide}}$ would be greater than that of the added AT β ³²P.

In a representative experiment, shown in Table 2, myokinase and AT β ³²P were dialysed in the presence of 0.1M-KCl, the latter substance being employed to prevent a Donnan distribution of nucleotide on either side of the membrane. At this concentration KCl does not inhibit myokinase, whereas M-KCl almost entirely inhibits the enzyme (Bowen & Kerwin, 1954). It can be seen that the enzyme was not completely separated from the added nucleotide, since the extinction at 260 m μ was always greater than at 280 m μ . The counts/ N_{260} ratio for the inner, enzyme-containing solution remained similar to that of the AT β ³²P standard, within the limits of experimental error due to pipetting, counting and spectrophotometric measurement, until the third dialysis. After this, the ratio fell below that of the standard so that the nucleotide in the enzyme solution must have consisted largely of ADP, formed by the gradual hydrolysis of ATP.

Table 2 also shows that the addition of *p*-chloromercuribenzoate caused a more rapid loss of radioactivity from the enzyme solution during dialysis.

It was observed that at the termination of the above-mentioned experiment (which continued for 9 days), a small protein precipitate had appeared in the membrane tubes, and only 20–50 % of the expected enzyme activity could be recovered in tubes 1–3. In the tubes containing *p*-chloromercuribenzoate the enzyme was entirely inactive, but 60–75 % of the expected activity could be assayed after activation with cysteine (Callaghan, 1957b).

After dialysis, the solutions were analysed chromatographically in the presence of 'carrier' amounts of ATP, ADP, AMP and PI, and the areas counted. Table 3 shows the results of this analysis. The areas on the starting line of the chromatogram

Table 2. *Dialysis of myokinase after addition of adenosine [³²P]triphosphate*

Dialysis was carried out in collodion tubes coated with cytochrome c. Constituents present in tubes 1-3 at zero time were: 5.15 mg. of pure myokinase; 0.385 mm.-ATP (containing $\text{ATP-}^{32}\text{P}$); 15 mm.-tris buffer, pH 7.5; 2 mm.- MgCl_2 ; 0.1 M-KCl; 10 mg. of neutralized cysteine hydrochloride. Total volume, 4 ml. Tubes pCMB1 and pCMB2 contained 0.84 mm.-p-chloromercuribenzoate initially, in addition to the above constituents, but contained no cysteine. Each tube was dialysed separately at 5-8° against 8 ml. of 0.1 M-KCl containing 15 mm.-tris buffer, pH 7.5, and subsequently against two lots of 200 ml. of buffered KCl solution. Preliminary experiments with the coated collodion tubes showed that dialysis equilibrium for ATP was reached in 48-55 hr. The value of N_{280} was calculated from equation (9) and the counts/ N_{280} ratio was estimated for standard solutions of $\text{ATP-}^{32}\text{P}$, which were counted with each set of samples taken.

(a) *First dialysis (against 8 ml. of buffered KCl soln.)*. Samples (0.1 ml.), taken from inside and outside solutions, were diluted to 2 ml. for spectrophotometry and then to 10 ml. for counting at the stated time intervals.

Tube	20 hr.			48 hr.			68 hr.				
	E_{280}	E_{280}	$\frac{\text{Counts/}}{5 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$	E_{280}	E_{280}	$\frac{\text{Counts/}}{5 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$	E_{280}	E_{280}	$\frac{\text{Counts/}}{5 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$		
1	0.150	0.056	1870	1.51	0.104	0.038	1551	0.103	0.035	1462	1.76
2	0.135	0.055	1840	1.72	0.109	0.044	1465	0.106	0.038	1475	1.65
3	0.127	0.058	1702	1.79	0.110	0.048	1485	0.109	0.043	1520	1.72
pCMB1	0.165	0.058	1957	—	0.134	0.045	1523	0.125	0.040	1500	—
pCMB2	0.193	0.080	1916	—	0.160	0.063	1061	0.155	0.060	1578	—
ATP standard	0.161	—	3023	1.87	0.161	—	2779	0.161	—	2774	1.72

(b) *Second dialysis*. The tubes were rapidly rinsed on the outside with water, and each tube was dialysed against 200 ml. of buffered KCl solution. After 67 hr. samples (0.5 ml.) were diluted to 2.5 ml. for spectrophotometry and then to 10 ml. for counting.

(c) *Third dialysis*. The tubes were dialysed against 200 ml. of fresh buffered KCl solution. After 48 hr. samples (1.0 ml.) were taken, diluted to 2.5 ml. for spectrophotometry and then to 10 ml. for counting.

Tube	$10^{-4} \times \frac{\text{Counts/}}{20 \text{ min.}} \times \frac{N_{280}}{N_{280}}$			$10^{-4} \times \frac{\text{Counts/}}{20 \text{ min.}} \times \frac{N_{280}}{N_{280}}$			$10^{-4} \times \frac{\text{Counts/}}{20 \text{ min.}} \times \frac{N_{280}}{N_{280}}$		
	E_{280}	E_{280}	$\frac{\text{Counts/}}{20 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$	E_{280}	E_{280}	$\frac{\text{Counts/}}{20 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$	E_{280}	E_{280}	$\frac{\text{Counts/}}{20 \text{ min.}} \times 10^{-4} \times \frac{N_{280}}{N_{280}}$
1	0.059	0.045	1414	5.1	0.196	0.161	2928	3.53	—
2	0.095	0.081	2167	5.9	0.205	0.173	2933	3.58	—
3	0.083	0.070	1754	5.35	0.280	0.229	4260	3.6	—
pCMB1	0.045	0.030	570	—	0.073	0.063	1019	—	—
pCMB2	0.108	0.088	634	—	0.154	0.142	764	—	—
AT ³² P standard	0.129	—	7146	5.6	0.129	—	6361	4.92	—

contained the myokinase protein, as well as a small amount of material, presumably nucleotide, which gave a dark spot when illuminated by the 2537 Å Hg line.

Since prolonged dialysis of myokinase led to a gradual inactivation of the enzyme, more rapid separation of the enzyme from added $\text{ATP}\gamma^{32}\text{P}$ was undertaken, the zone-electrophoresis apparatus of Porath (1954) being used. Figs. 1 and 2 show representative experiments in which partial and complete separations were achieved. In Fig. 1 it is shown that the counts/ N_{260} ratio fell sharply from the standard value of the nucleotide fractions to a low value in the enzyme-containing fractions; after complete separation (Fig. 2) the protein contained some firmly bound nucleotide, but gave no significant count above the background count. From these results it is inferred that the nucleotide associated with the enzyme was AMP (since this compound would be negligibly labelled with ^{32}P). This trace of AMP presumably arose from the slow hydrolysis of ATP, coupled with enzyme activity. In the experiment given in Fig. 2, the $\text{ATP}\gamma^{32}\text{P}$ migrated completely out of the electrophoresis column into the tank of buffer, where its presence was detected by the radioactivity of the buffer.

Absorption spectra of myokinase and adenosine triphosphate in solution. In the above-mentioned experiment, it was observed that nucleotide appeared in the enzyme fractions after removal of ATP by electrophoresis. The absorption spectra of these solutions were compared with those of mixtures of myokinase and nucleotides at known concentrations.

Fig. 3 shows the spectrum of a representative myokinase preparation used for these studies; a similar spectrum has been published by Noda &

Kuby (1957*b*) for crystalline myokinase. A well-defined trough is present in the spectrum at 250 $\text{m}\mu$, the ratio E_{279}/E_{250} for our preparation being 5.6, whereas that for the preparation of Noda & Kuby is 2.3, as measured from their published spectrum. The presence of traces of nucleotide fills the trough at 250 $\text{m}\mu$ and, at higher concentrations, shifts the maximum from 279 $\text{m}\mu$ to the region of 265 $\text{m}\mu$. The resulting spectrum, however, is purely the sum of the spectra of the two components. The spectrum of a myokinase fraction (fraction 19 in Fig. 2) which contained traces of bound nucleotide, presumably AMP, after electrophoretic removal of ATP showed that the

Table 3. *Chromatographic separation of radioactive material in dialysed enzyme nucleotide solution*

Specimens (0.2 ml.) from each tube from the dialysis experiment (Table 2) were spotted on to Whatman no. 1 paper. On each spot 5 $\mu\text{l.}$ of 0.02 M-ATP, -ADP, -AMP and -PI was superimposed to act as carriers for traces of radioactive phosphates. After chromatography (Krebs & Hems, 1953) the areas were identified by viewing in ultraviolet light, and PI was located by the molybdate spray and H_2S (Hanes & Isherwood, 1949). The total areas were cut as circles for counting; 20 min. counts of each area are given. *p*CMB 1, *p*CMB 2: tubes contained *p*-chloromercuribenzoate initially (see Table 2).

Specimen	PI	ATP	ADP	AMP	Starting spot (protein)
1	68	11	0	0	461
2	18	23	0	0	301
3	134	31	32	0	666
<i>p</i> CMB 1	7	5	0	0	13
<i>p</i> CMB 2	41	60	—	—	23

Fig. 1. Partial separation of myokinase from $\text{ATP}\gamma^{32}\text{P}$ by zone electrophoresis. The column of the electrophoresis apparatus contained borate-phosphate buffer, pH 8.4, μ 0.05. The following mixture was introduced as a zone into the column in a total volume of 6.01 ml.: 15 mg. of myokinase (previously dialysed against buffer); 1 ml. of 10 mM- $\text{ATP}\gamma^{32}\text{P}$; 15 mg. of cysteine hydrochloride neutralized to pH 8.4; 0.01 ml. of M-MgCl₂. After electrophoresis for 9 hr. at 8°, with a current of 22 mA (140 V, D.C.), 10 ml. fractions were collected from the column. ○, $10^{-4} \times (\text{Counts}/N_{260} \text{ ratio})$; ●, extinction at 260 $\text{m}\mu$; ■, extinction at 280 $\text{m}\mu$; □, enzyme activity. The arrow indicates the direction of migration towards the cathode.

ratio E_{270}/E_{250} was altered to 1.0, and an absorption maximum was present at 265 m μ .

Previous experience has shown that the enzyme binds traces of nucleotides very tenaciously during the process of purification. It is not suggested that this type of binding is necessarily related to the formation of enzyme-substrate complexes.

Fig. 2. Total separation of myokinase-nucleotide complex from $\text{ATP-}^{32}\text{P}$ by zone electrophoresis. The column contained borate-phosphate buffer, pH 8.4, μ 0.05. The following mixture was introduced into the column as a zone in a total volume of 5.01 ml.: 10 mg. of myokinase (previously dialysed against buffer); 1 ml. of 10 mM- $\text{ATP-}^{32}\text{P}$; 15 mg. of cysteine hydrochloride neutralized to pH 8.4; 0.01 ml. of M-MgCl_2 . After electrophoresis for 27.75 hr. at 8°, with a current of 21 mA (approx. 140 v, d.c.), 7 ml. fractions were collected from the column. ●, Extinction at 260 m μ ; ○, extinction at 280 m μ ; ■, enzyme activity. The arrow indicates the direction of migration towards the cathode.

Fig. 3. Absorption spectrum of myokinase (0.51 mg./ml.) in 0.01 M-phosphate buffer, pH 7.0 (see text).

Kinetic studies

Inhibitor constants of adenosine tri- and mono-phosphate and p-chloromercuribenzoate. A comparison of the initial velocities of the forward and

Fig. 4. Progress curve of reaction $\text{ATP} + \text{AMP} \rightarrow 2\text{ADP}$ at 30°. The constituents present were: 6 mM-ATP; 6 mM-AMP; 30 mM-tris buffer, pH 7.5; 2 mM- MgCl_2 ; 1 $\mu\text{g.}$ of myokinase. Volume at zero time, 1.6 ml. Samples were removed at times indicated and treated with 0.1 vol. of 30% (w/v) trichloroacetic acid.

Fig. 5. Representative Lineweaver-Burk plots of initial velocity of myokinase reactions at 30° by the technique of Callaghan (1957b), with varied concentration of ADP, and inhibition by mM-ATP and 0.5 mM-AMP (competitive) and 0.015 μM -p-chloromercuribenzoate (pCMB) (non-competitive). At zero time, in a total volume of 1.6 ml., the constituents present were: ADP (as indicated in the figure); 0.03 M-tris buffer, pH 7.5; 2 mM- MgCl_2 ; 0.5 $\mu\text{g.}$ of myokinase. Inhibitors were present in the reaction mixtures as indicated. Initial velocity in μmoles of ADP dismutated was calculated for 1 min. of reaction (v), at each concentration of ADP (S). Intercept for ADP control plot at $-1/K_m$ gives a K_m value of 1.58 mM.

reverse reactions catalysed by myokinase showed that whereas the velocity of the forward reaction ($2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$) decays rapidly with time (Callaghan, 1957b), the velocity of the reverse reaction is more constant (Fig. 4) and proceeds at 25–30 % of the initial rate of the forward reaction when the concentrations of ATP and AMP are separately equal to that of the ADP used in the forward reaction. These differences are believed to be due partly to the inhibitory effect of AMP (Colowick & Kalckar, 1943; Oliver, 1955); Fig. 5 shows that both AMP and ATP competitively inhibit the forward reaction, having enzyme-inhibitor dissociation constants (K_i) of 0.5 mM and 0.32 mM respectively, as calculated according to Massart (1950). Thus the formation of these substances by the forward reaction is followed by deceleration.

p-Chloromercuribenzoate inhibited myokinase non-competitively (Fig. 5). At a concentration of 8 mM-ADP, equimolar amounts of *p*-chloromercuribenzoate and myokinase (0.015 μM) showed 50 % of the control activity. Addition of cysteine (5 mg./ml.) entirely abolished this inhibition. The K_i value for *p*-chloromercuribenzoate was calculated from the formula $v/v_i = 1 + ([I]/K_i)$ (Massart, 1950) to be 0.015 μM .

It should be pointed out that myokinase has two SH groups (Callaghan, 1957a) and that this calculation, taken from a single measurement, is only satisfactory if it is assumed that (a) only one SH group need be blocked to produce inactivation and (b) the same group has a K_i very much smaller

than the other SH group. If, for example, both groups had equal K_i values and both needed to be blocked for inactivation, total binding of the mercurial compound would result in the above case, giving 50 % inhibition, but with $K_i \rightarrow 0$. A more thorough study is required to clarify these points.

Effect of other nucleotides and aromatic compounds on myokinase activity. At concentrations of 0.5 mM, inosine monophosphate, uridine monophosphate, cytidylic acid and guanylic acid gave no detectable inhibition, with concentrations of ADP ranging from 0.8 to 8.0 mM. Neither aniline nor benzoate (0.5 mM) showed any inhibition in the presence of 8 mM-ADP. At concentrations of mM no inhibition was produced by inosine monophosphate, uridine monophosphate, cytidylic acid or adenosine (Fig. 6), but adenosine monosulphate was shown to be a competitive inhibitor

$$(K_i = 0.0186 \text{ mM}).$$

Cross-reactions between nucleotides catalysed by traces of contaminating enzymes in the myokinase preparation. Previous studies (Callaghan, 1957b) showed that the myokinase preparation was free of certain enzymes which might act on nucleotides. In Table 4 it is shown that the only reaction which took place was inosine triphosphate + AMP $\rightarrow$ inosine diphosphate + ADP, and the resulting ADP was dismutated by the excess of myokinase to give rise to ATP (reaction mixture 2). This indicated the presence of a trace of nucleoside monophosphate kinase activity (sufficient to cause the disappearance of approximately 0.025 μmole of inosine triphosphate/min.) in 16 μg . of protein. Since 1 μg . of myokinase transforms 0.66 μmole of ATP or AMP/min. the nucleoside monophosphate kinase activity would cause the removal of AMP (or inosine triphosphate) at a rate of 0.24 % of that of the myokinase, and no attempt was made to remove this trace of contaminant for the kinetic studies presented here. In reaction mixture 2, the presence of the contaminating enzyme caused a shift in the equilibrium of the myokinase reaction.

The equilibrium constant

$$K = \frac{[\text{ATP}][\text{AMP}]}{[\text{ADP}]^2} = 0.444$$

at pH 7.4 has been reported by Eggleston & Hems (1952) and Callaghan (1957b). In reaction mixture 2, $K = 0.364$. This effect has been noted by Gibson, Ayengar & Sanadi (1956). Nucleoside diphosphate kinase was absent from the preparation (mixture 1) and the myokinase equilibrium gave $K = 0.42$. In this mixture, the small amount of formed AMP was apparently not acted on by the monophosphate kinase during the incubation period. No reaction between ATP and either uridine di- or mono-phosphate was observed

Fig. 6. Effect of nucleotides and adenine monosulphate on myokinase action at 30°. At zero time, in a total volume of 2.0 ml., the constituents present in the reaction mixtures were: 1 μg . of myokinase; 30 mM-tris buffer, pH 7.5; 2 mM-MgCl₂; ADP and nucleotides or adenine monosulphate as indicated. Reciprocal of velocity ($1/v$) in μmoles of ADP dismutated. Reciprocal of molar ADP concentration ($1/S$). The lower line shows the plot of the control reactions with ADP (points not shown). □, 0.0191 mM-Adenine monosulphate; ○, mM-adenosine; ▲, mM-inosine triphosphate; ■, mM-cytidylic acid; ●, mM-uridine monophosphate.

Table 4. *Cross-reactions between adenosine nucleotides and other nucleotides catalysed by the myokinase preparation*

In a total volume of 0.6 ml. the constituents present in each reaction mixture at zero time were: 16 μ g. of enzyme; 20 mM-tris buffer, pH 7.5; 1.5 mM-MgCl₂; nucleotides as indicated in the Table. Incubation was at 30° and samples (0.05 ml.) were withdrawn at the times indicated for treatment with 0.1 vol. of 30% (w/v) trichloroacetic acid. Nucleotides were separated chromatographically (Krebs & Hems, 1953) and estimated by phosphate analysis. All figures in the Table give the total amount of each nucleotide (in μ moles) calculated for the zero-time volume (0.6 ml.). ITP, Inosine triphosphate; IDP, inosine diphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate.

Reaction mixture	...	(1) ITP* + ADP			(2) ITP + AMP			(3) ATP + UMP		(4) ATP + UDP	
Time (min.)	...	0	2	30	0	2	30	0	30	0	30
Nucleotide											
ITP		0.58	0.56	0.56	0.55	0.50	0.13	—	—	—	—
IDP		0.38	0.37	0.38	0.32	0.36	0.76	—	—	—	—
ATP		—	0.70	0.68	—	—	0.14	1.27	1.31	1.40	1.50
ADP		2.4	1.10	1.05	—	0.08	0.27	0.06	0.08	0.11	0.13
AMP		0.1	0.71	0.69	2.32	2.24	1.90	—	—	—	—
UDP		—	—	—	—	—	—	—	—	0.38	0.39
UMP		—	—	—	—	—	—	2.10	2.17	—	—

* It should be noted that the ITP preparation contained about 40% of IDP.

Fig. 7. Effect of Mg²⁺ ion concentration on the forward reaction at 30°. At zero time, the constituents present, in a total volume of 1.6 ml., were: 1 μ g. of enzyme; 30 mM-tris buffer, pH 7.5; 80 mM-ADP. After 1 min. the reaction was stopped by addition of 0.1 vol. of 30% (w/v) trichloroacetic acid.

(reaction mixtures 3 and 4). The latter reaction is catalysed by the monophosphate kinase in yeast (Lieberman, Kornberg & Simms, 1954; Munch-Petersen, 1954), but this preparation (from rabbit muscle) was inactive towards uridine monophosphate.

Activation by magnesium. In the absence of Mg²⁺ ions no myokinase activity could be observed; thus the enzyme appeared to be free of bound magnesium. Fig. 7 shows that optimum activity was obtained at magnesium concentrations similar to that of the substrate concentrations. These results are in agreement with the finding of Kalckar (1943) that, at pH 7.5, enzyme activity is greatest in 0.01M-MgCl₂, and appear to bear out the suggestion of Bowen & Kerwin (1954) that myokinase reacts with the magnesium complexes of adenosine phosphates. Kuby, Noda & Lardy (1954) have indicated that creatine phosphokinase also reacts with the magnesium complexes of ATP and ADP.

Inhibition by fluoride. The inhibitory effect of fluoride on myokinase activity in various tissues has been reported by Kotel'nikova (1950), Barkulis & Lehniger (1951), Leuhardt & Bruttin (1952) and Siekevitz & Potter (1953). The writers found that 0.02M-sodium fluoride inhibits the forward reaction by 62% and the reverse reaction by 56% at pH 7.5 in the presence of 2 mM-MgCl₂. Kotel'nikova reported that 0.02M-fluoride caused 43–48% inhibition (presumably of the forward reaction) of myokinase activity in extracts of rabbit muscle, heart and liver, and also noted that phosphate did not potentiate this inhibition. We have not studied the action of phosphate in fluoride inhibition.

Kinetics of the reverse reaction; theoretical introduction. In determining initial velocities for the reaction 2 ADP $\rightarrow$ ATP + AMP, it is necessary to take account of the rapid inhibition produced by the accumulated products. A simple best straight line connecting the experimental values for the velocity at different times is incorrect since the actual reaction course always shows a curvature concave towards the time axis. To obtain the initial velocity the tangent to the curve at time ≈ 0 must be obtained. The mirror method (Callaghan, 1957b) was used with this object.

In Fig. 5 it was shown that the reciprocal plot for the dismutation of ADP is linear, which suggested that the velocity of the forward reaction was a simple function of the ADP concentration. This implies that only one site is present on myokinase for the catalytic adsorption of ADP, since if two molecules of ADP were activated simultaneously, the following equation [see Dixon & Webb (1958)] will apply:

$$\frac{1}{v} = \frac{1}{V_{\max.}} \left[1 + \frac{K_1}{A_0} + \frac{K_2}{B_0} + \frac{K_1 K_2}{A_0 B_0} \right],$$

where v is the velocity of reaction measured, $V_{\max}$ is the maximum velocity and K_1 and K_2 are the respective K_m values for substrates A and B, which are present at the concentrations A_0 and B_0 . In the reaction $2 \text{ ADP} \rightarrow \text{ATP} + \text{AMP}$, $A_0 = B_0 = [\text{ADP}]$

$$\text{and } \frac{1}{v} = \frac{1}{V_{\max}} \left[1 + \frac{K_1 + K_2}{[\text{ADP}]} + \frac{K_1 K_2}{[\text{ADP}]^2} \right]. \quad (8)$$

Thus on plotting $1/v$ against $1/[\text{ADP}]$, a curvature convex towards the $1/[\text{ADP}]$ axis would be observed. The departure from linearity would be due to the square term in $[\text{ADP}]$.

In the range of concentrations which we could use no such deviation was observed, or at least the deviation was smaller than the experimental error. This did not rule out the possibility that the value for K_2 was so small that, even at the lowest concentration of substrate used, the second site was fully saturated with ADP.

If E is the vertical height of the usual reciprocal plot above infinite substrate concentration and if the linear law is followed with an experimental error smaller than the fractional departure from linearity, ΔE , down to a concentration $[\text{ADP}]_{\min.}$, we have

$$\frac{\frac{K_1 K_2}{[\text{ADP}]_{\min.}^2}}{1 + \frac{K_1 + K_2}{[\text{ADP}]_{\min.}}} < \frac{\Delta E}{E}.$$

$$\text{Thus } \frac{K_1 K_2}{[\text{ADP}]_{\min.}} < \frac{\Delta E}{E} ([\text{ADP}]_{\min.} + K_1 + K_2),$$

$$\text{since } K_2 \ll K_1 \gg [\text{ADP}]_{\min.}$$

$$K_2 < \frac{\Delta E}{E} [\text{ADP}]_{\min.} \quad (9)$$

Equation (9) gives the relation between the experimental error and the maximum possible value for K_2 .

In the reciprocal plot for ADP given in Fig. 5, E was 2.35; the experimental error involved in the estimation of ADP by our methods is $\pm 3\%$. At zero time $1.17 \mu\text{moles}$ of ADP was present in the reaction mixture. After 1 min. of reaction, $0.3 \mu\text{mole}$ of ADP had been consumed ($1/v = 3.35$). Thus $0.87 \mu\text{mole}$ of ADP remained for estimation; 6% of this is $0.05 \mu\text{mole}$. This error could vary the estimation of the ADP content after 1 min. from, say, 0.87 to $0.82 \mu\text{mole}$, thus varying $1/v$ from 3.35

($v = 0.3 \mu\text{mole/min.}$) to 4.0 ($v = 0.25 \mu\text{mole/min.}$). The fractional departure, $\Delta E/E$, in the estimated height would be $0.65/2.35 = 0.276$. From equation (9) $K_2 < 0.276 \times 7.3 \times 10^{-4} \text{ M-ADP}$. Therefore the maximum possible value for K_2 is 0.2 mM . With such a low value of K_2 it would be expected that ADP would be a powerful inhibitor of the reverse reaction, as it would compete for a site on the enzyme occupied by either ATP or AMP. This is implicit in the principle of microscopic reversibility, which holds that the intermediate complex formed at the surface of a catalyst in a reaction of the type $A + B \rightleftharpoons \text{intermediate complex} \rightleftharpoons C + D$ is the same for the forward as for the reverse process.

Assuming the existence of two sites on the enzyme, the inhibitory effect of ADP at low concentrations (of the order of K_2) on the reaction $\text{ATP} + \text{AMP} \rightarrow 2 \text{ ADP}$ is given by equations (10) and (10a) below.

The following general considerations will also apply if myokinase possesses separate sites on the active centre for the two substrates ATP and AMP.

Let the enzyme have sites 1 and 2 for the specific adsorption of substrates 1 and 2 respectively with primary equilibrium constants $K_{1,1}$ and $K_{2,2}$. It is also possible that substrate 1 will be adsorbed at site 2, and substrate 2 at site 1, with constants $K_{1,2}$ and $K_{2,1}$ respectively. It is to be expected that these latter constants will be considerably larger than their respective primary constants, otherwise the efficiency of the enzyme will be very much impaired.

The probability that molecules of the enzyme will form the primary complexes with the substrates is the probability of finding substrate 1 at site 1, and substrate 2 at site 2, simultaneously. The fraction of the enzyme molecules having substrate 1 at site 1 in the absence of substrate 2 is

$$f_1 = \frac{S_1}{K_{1,1} + S_1} = \frac{S_1/K_{1,1}}{1 + S_1/K_{1,1}},$$

where S_1 is the concentration of substrate 1. In the presence of substrate 2, the probability of finding substrate 1 at site 1 is

$$F_1 = \frac{S_1/K_{1,1}}{1 + \frac{S_1}{K_{1,1}} + \frac{S_2}{K_{2,1}}},$$

where S_2 is the concentration of substrate 2.

$$\begin{aligned} \frac{1}{v} &= \frac{1}{V} \left[\left(1 + \frac{K_{\text{ATP}}}{[\text{ATP}]} \right) \left(1 + \frac{K_{\text{AMP}}}{[\text{AMP}]} \right) \right] \\ &= \frac{1}{V} \left[1 + \frac{K_{\text{ATP}}}{[\text{ATP}]} + \frac{K_{\text{AMP}}}{[\text{AMP}]} + \frac{K_{\text{ATP}} \cdot K_{\text{AMP}}}{[\text{ATP}] [\text{AMP}]} \right] \end{aligned} \quad (10)$$

$$\text{and } \frac{1}{vi} = \frac{1}{V} \left\{ 1 + \left[\frac{K_{\text{ATP}}}{[\text{ATP}]} + \frac{K_{\text{AMP}}}{[\text{AMP}]} + \frac{K_{\text{ATP}} \cdot K_{\text{AMP}}}{[\text{ATP}] [\text{AMP}]} \right] \left[1 + \frac{[\text{ADP}]}{K_2} \right] \right\}. \quad (10a)$$

Similarly,

$$F_2 = \frac{S_2/K_{2,2}}{1 + \frac{S_2}{K_{2,2}} + \frac{S_1}{K_{1,2}}}$$

The probability of the simultaneous presence of substrates 1 and 2 in their primary places is $F_1 F_2$, which is also the ratio v/V . Since this will be the fraction of maximum velocity, it can be seen that V is reached when $F_1 F_2 \rightarrow 1$. Thus

$$\frac{V}{v} = \frac{1}{F_1 F_2} = \left\{ 1 + \frac{K_{2,2}}{S_2} \left[1 + \frac{S_1}{K_{1,2}} \right] \right\} \times \left\{ 1 + \frac{K_{1,1}}{S_1} \left[1 + \frac{S_2}{K_{2,1}} \right] \right\}. \quad (11)$$

An equivalent equation has been derived by Laidler & Socquet (1950). To observe experimentally the effects of the double affinity of substrate 1 for sites 1 and 2, let $S_2 = K_{2,2}$ ($K_{2,2}$ being the Michaelis constant for its own site). At this concentration, the ratio $S_2/K_{2,1}$ can be neglected, since $K_{2,2} \ll K_{2,1}$. Equation (11) then becomes

$$\frac{V}{v} = \left[2 + \frac{S_1}{K_{1,2}} \right] \left[1 + \frac{K_{1,1}}{S_1} \right] = \left[2 + \frac{S_1}{K_{1,1}} \left(\frac{K_{1,1}}{K_{1,2}} \right) \right] \left[1 + \frac{K_{1,1}}{S_1} \right].$$

Let $K_{1,1}/S_1 = x$ and $K_{1,1}/K_{1,2} = R$, then

$$\frac{V}{v} = \left[2 + \left(\frac{K_{1,1}}{K_{1,2}} \right) x \right] [1 + x] = 2(1 + x) + R \left(1 + \frac{1}{x} \right). \quad (12)$$

Fig. 8. Plot of V/v against $K_{1,1}/S_1$ for various values of R (see text).

Fig. 8 shows the family of curves obtained by plotting V/v against $K_{1,1}/S_1$ for different values of R , as calculated from equation (12).

It is clear that as the substrate concentration is increased the velocity increases to a maximum. Beyond this point the velocity decreases as the substrate begins to saturate the second site on the enzyme, with resulting inhibition.

Such an effect may be tested for experimentally by plotting $1/v$ against $1/S$ for each substrate, while the other substrate is present at a concentration near its K_m value (that is, its primary association constant).

Kinetics of the reverse reaction; results. (a) Inhibition of reverse reaction by ADP.

At pH 7.5 and 30°, 0.5 mM-ADP ([ADP] in equations 10 and 10a) caused 24% inhibition of the reverse reaction in the presence of 0.6 mM-AMP ([AMP]), 0.6 mM chromatographically pure ATP ([ATP]), 0.002M-MgCl₂ and 75 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer. From equations (10) and (10a) it was calculated that for a value of $K_2 = 0.2$ mM-ADP (derived from equation 9) an inhibition of 61% was to be expected, given that $K_{ATP} = 0.3$ mM and $K_{AMP} = 0.5$ mM (these values are given in the results below). If the value $K_2 = 1.6$ mM-ADP (Callaghan, 1957b) was used instead, the theoretical inhibition would be 17%.

The observed inhibition of 24% more closely approximates to the last-mentioned value, and the discrepancy may be due to our inability to measure the initial reaction velocities with greater precision. This evidence suggests that it is unnecessary to assume the existence of a second binding constant of low value (K_2) for ADP.

(b) Measurement of K_m values for ATP and AMP.

Figs. 9 and 10 show the results of experiments where the velocity of the reverse reaction was measured at various concentrations of one substrate, while the other substrate was initially present at a concentration near its K_m value. The reciprocal plots showed K_m values for ATP and AMP of 0.3 mM and 0.5 mM respectively. In both figures the highest substrate concentration was such that $K_{1,1}/S_1$ was 0.05 (compare with Fig. 8). It will be seen that there was no decrease in reaction velocity at the highest substrate concentrations.

Thus if two sites are present on the enzyme, each site is highly specific for its own substrate. In numerical terms, $K_{1,2}$ must be at least one hundred times as large as $K_{1,1}$ (see Fig. 8, for curve where $R = 0.01$).

Since it has already been indicated that there is probably only one K_m value for ADP, the simplest explanation of these facts is that myokinase has one site which adsorbs all three substrates in turn.

Fig. 9. Lineweaver-Burk plot for the velocity of myokinase reverse reaction at 30° , when the concentration of ATP is varied while AMP is present at its K_m concentration. At zero time, in a total volume of 1.6 ml., the constituents present in the reaction mixtures were: 0.5 mM-AMP; ATP varied from 7.2 mM to 0.072 mM, as indicated on the Figure; 2 mM-MgCl₂; 30 mM-tris buffer, pH 7.5; 1 μ g. of myokinase. After incubation for 2 min. the reaction was stopped by the addition of 0.1 vol. of 30% (w/v) trichloroacetic acid. Ordinate shows reciprocal of velocity ($1/v$) in μ moles of ATP disappearing and the abscissa shows reciprocal of molar ATP concentration ($1/S$).

Fig. 10. Lineweaver-Burk plot for the velocity of myokinase reverse reactions at 30° when the concentration of AMP is varied while ATP is present at a concentration near its K_m value. At zero time, in a total volume of 1.6 ml., the constituents in the reaction mixtures were: 0.72 mM-ATP; AMP varied from 10 mM to 0.1 mM as indicated in the Figure; 2 mM-MgCl₂; 30 mM-tris buffer, pH 7.5; 1 μ g. of myokinase. After incubation for 2 min., the reaction was stopped by the addition of 0.1 vol. of 30% (w/v) trichloroacetic acid. Ordinate shows reciprocal of velocity ($1/v$) in μ moles of ATP disappearing, and the abscissa shows reciprocal of molar AMP concentration ($1/S$).

DISCUSSION

From the results presented in this paper it appears that myokinase does not react with its substrates to form an intermediate phosphorylated enzyme, such as has been described by Sidbury & Najjar (1957) for phosphoglucumutase. Whereas these authors crystallized the phosphorylated phosphoglucumutase, the crystalline preparation of myokinase (Noda & Kuby, 1957b) contained only 0.044% of phosphorus in ashed specimens. This is less than 1 g. of phosphorus/21 000 g. of enzyme, and, as was pointed out earlier, traces of nucleotide are strongly bound to the enzyme. This may account for the traces of phosphorus present.

Myokinase belongs to that group of phosphokinases previously studied by Cohn (1953, 1954) and Harrison, Boyer & Falcone (1955), which show similar characteristics of phosphate transfer with respect to the position of cleavage of the phosphate bond. Within this group of phosphokinases, unequivocal evidence for the formation of a phosphorylated enzyme intermediate has not been obtained. Detailed studies of the mechanism of action of pyruvate kinase (Boyer & Harrison, 1954), acetokinase (Rose, Grunberg-Manago, Korey & Ochoa, 1954) and glutamine synthetase (Kowalsky, Wyttenbach, Langer & Koshland, 1956; Boyer, Koeppe & Luchsinger, 1956) have indicated that it is not essential to postulate the formation of this type of intermediate and, for pyruvate kinase, the evidence is against it.

The detailed mechanism of myokinase action is envisaged as follows. The enzyme has a single site which binds specifically one molecule of substrate. On contact with another substrate molecule reaction takes place (equations 4a and 4b). This contact of the E-S complex with the second substrate molecule may be envisaged as involving a simple collision or, more likely, a binding with Michaelis constant much lower than the one of the first molecule and also involving a much smaller interaction energy and dependent on the previous formation of the E-S complex.

The fact that AMP has approximately equal K_i and K_m deprives this nucleotide of any specific significance as an inhibitor. However, the results obtained in the dialysis and electrophoresis experiments indicate that strong binding of AMP by the enzyme may occur. The relation of this to the specific catalytic binding is not clear.

The following facts summarize the case for a one-site theory of myokinase action: (1) There appears to be only one binding constant for ADP, and calculation indicates that $K_m \text{ ADP} = K_i \text{ ADP}$. (2) Whereas both ATP and AMP inhibit the reaction $2 \text{ ADP} \rightarrow \text{ATP} + \text{AMP}$ in a competitive manner they do not inhibit each other's binding in

the reverse reaction. There are no secondary binding constants for ATP and AMP; the primary association constants are the same, within experimental error, as their inhibitor constants.

$$K_m \text{ ATP} = 0.3 \text{ mM}, K_i \text{ ATP} = 0.32 \text{ mM},$$

$$K_m \text{ AMP} = 0.5 \text{ mM}, K_i \text{ AMP} = 0.5 \text{ mM}.$$

The potent non-competitive inhibition of myokinase by *p*-chloromercuribenzoate, and the action of this inhibitor in causing the loss of substrate from solutions of enzyme during dialysis, indicate that the SH groups of the enzyme are essential for catalytic activity. It is possible that the attachment of *p*-chloromercuribenzoate to SH groups in the vicinity of the active centre of the enzyme may sterically hinder the adsorption of substrate.

Since none of the other nucleotides tested, with the exception of adenosine monosulphate, inhibited the enzyme, it seems that the intact adenosine phosphate molecule is necessary for attachment to the enzyme. Inosine monophosphate, for example, differs only from AMP by the lack of an amino group on the purine ring, whereas adenosine is AMP minus the phosphate.

The inhibition by adenosine monosulphate suggests that the sulphate radical attaches to a group which is also available to the phosphate group. The amino group on the purine ring was previously stated to be necessary for the binding of ATP and ADP to myokinase by Bendall (1954), and Krebs & Hems (1953) reported that myokinase did not catalyse transphosphorylations involving inosine phosphates.

SUMMARY

1. A phosphorylated enzyme intermediate could not be detected when purified myokinase was incubated with adenosine triphosphate (ATP) and $\text{ATP}\gamma^{32}\text{P}$, comparable concentrations of enzyme and substrate being used, and the enzyme was separated from the substrate by dialysis or zone electrophoresis.

2. The K_m and K_i values for the substrate ATP are 0.3 mM and 0.32 mM respectively. For adenosine diphosphate (ADP), $K_m = K_i = 1.58 \text{ mM}$. For adenosine monophosphate (AMP), the K_m and K_i values are 0.5 mM.

3. Kinetic studies showed that ATP and AMP inhibited the forward reaction competitively.

4. A one-site theory for the mechanism of action of myokinase is proposed and discussed, evidence being brought to show that there is no demonstrable second site on the enzyme for any of the substrates. A theoretical treatment is given for the detection of secondary association constants.

5. *p*-Chloromercuribenzoate inhibits myokinase non-competitively ($K_i = 0.015 \mu\text{M}$) and adenosine

5'-monosulphate inhibits myokinase competitively ($K_i = 18.6 \mu\text{M}$).

6. Other nucleotides tested did not show detectable inhibition of myokinase, and aniline and benzoate were without effect. Apparently the amino and phosphate groups as well as the adenine ring are specifically required for enzyme activity, although the sulphate group may be substituted for phosphate for purposes of contact with the active centre.

7. A trace of nucleoside monophosphate kinase activity was detected in our myokinase preparations.

8. Myokinase is activated by Mg^{2+} ions. Optimum activation occurs when the Mg^{2+} ion concentration is of the same order as the substrate concentration (0.01M).

9. The enzyme is inhibited 56–62% by 0.02M-sodium fluoride.

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ADDENDUM

After this paper was written the recent article of Noda [*J. biol. Chem.* (1958) **232**, 237] came to our notice. An important point of difference from the kinetic data reported here was the value of the inhibitor constant for ADP reported by Noda as 0.33 mM, whereas ours appears similar to the $K_m = 1.6 \text{ mM}$. Moreover, Noda obtained a linear plot of $1/v$ against $1/[\text{ADP}]^2$. Such a linear plot would not fit either a one-site ($K_2 = 0$) or two equivalent sites ($K_1 = K_2$) theory as indicated by equation (8). However, over a restricted range of substrate concentration such a plot would be approximately linear where $K_1/\text{ADP} \gg 1$ if two sites of equal K_m were present. On the one-site theory, on the other hand, the plot of $1/v$ against $1/[\text{ADP}]^2$ would yield a curve concave towards the latter axis. In fact it appears to us that the curve of Fig. 6 (for AMP = 0) in Noda's paper shows such behaviour.

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α -1:4-Glucosans

10. GLYCOGEN STRUCTURE AND *RIGOR MORTIS* IN MAMMALIAN MUSCLES*

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Numerous chemical studies have demonstrated that glycogens from various sources may differ in such characteristics as molecular weight, average chain length and in the relative number of D-glucose residues in external and internal chains (Manners, 1957). That constitutional differences in the glycogen molecule have metabolic significance has been indicated by Stetten, Katzen & Stetten (1956), who showed that in the rat and rabbit the larger molecules of muscle glycogen are more reactive, and by Stetten & Stetten (1955), who found that D-glucose residues located peripherally in the chains have a higher rate of turnover than those more centrally situated. Another aspect of the apparent physiological inhomogeneity of

mammalian-muscle glycogen has arisen during investigations on post-mortem glycolysis, and on the concomitant onset of *rigor mortis*. In general, provided that pre-slaughter stress has not seriously depleted glycogen reserves, post-mortem glycolysis will continue until pH 5.4–5.5 is reached in the muscles, at which the enzyme systems producing lactic acid are inactivated and some residual glycogen may be found. At a final pH of 5.9, 0.6–1 % of glycogen frequently remains in the psoas and diaphragm muscles of the horse (Lawrie, 1955), and 0.6 % at a final pH of 6.1 in the sternocephalic muscle of the ox (Howard & Lawrie, 1957). This could signify that the glycogens remaining in such muscles after the completion of *rigor mortis* were either inaccessible or insusceptible to attack during post-mortem glycolysis, or that they might differ in

* Part 9, Eddy, Fleming & Manners (1958).