The Adenosine Triphosphate Inhibition of the Pyruvate Kinase Reaction and its Dependence on the Total Magnesium Ion Concentration

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1. The effects of ATP, PP_i and EDTA on the skeletal-muscle pyruvate kinase reaction at various concentrations of magnesium (where 'magnesium' refers to total Mg^{2+} , both free and in the form of complexes) were investigated. The reaction rate was determined as the amount of pyruvate formed in a recorded time of incubation. 2. At 44mM-magnesium the K_m values for ADP and phosphoenolpyruvate were unaltered by the presence of ATP up to 6.8mM in systems buffered with either tris-hydrochloric acid or glycylglycine-sodium hydroxide, but the K_m values were different in these systems. The K_m for one substrate was independent of the concentration of the second substrate. 3. At 10mM-magnesium in the tris-hydrochloric acid system ATP inhibited the reaction competitively with respect to ADP and phosphoenolpyruvate. In the glycylglycine-sodium hydroxide system the inhibition appeared to be non-competitive. At 10mM-magnesium the K_m values were lower than at 44mM-magnesium and dependent on the system used. 4. In the tris-hydrochloric acid system the reaction rate rose with increasing magnesium concentration up to a maximum at a concentration 10–20 times that of ADP. Further increase inhibited the reaction and at 44mM-magnesium the rate was 25–50% of its maximum. This inhibition paralleled that produced by increasing trimethylammonium chloride concentrations and was not due to a specific effect of the Mg^{2+} ion. 5. In the presence of 6.8mM-ATP no reaction occurred below 4–6mM-magnesium, and further increase apparently abolished the inhibition as the reaction rate increased and became equal to those obtained in the absence of ATP at 10–25mM-magnesium. Further increase in magnesium concentration gave reaction rates that were slightly higher in the presence of ATP than in its absence. The maximal rate in the presence of ATP was distinctly lower than in its absence. When 6.8mM- PP_i or 6.8mM-EDTA was present the variations in reaction rate with rising magnesium concentration were similar to that obtained in the presence of ATP below 6–8mM-magnesium but further increase in the magnesium concentration resulted in an increase in the rate up to a maximum comparable with that of the control. The effect of pure chelation was thus a displacement of the reaction maximum to higher magnesium concentrations without changing the maximal rate. When correction had been made for this effect, ATP gave inhibition at 44mM-magnesium that was competitive with respect to ADP (K_i $2.1 \times 10^{-2} M$). This degree of inhibition is far less than was reported earlier and its importance for the mechanism of the pyruvate kinase reaction is discussed.

Inhibition by ATP of the pyruvate kinase reaction:

was first observed by Meyerhof & Oesper (1949). This was later studied in detail by Reynard, Hass, Jacobsen & Boyer (1961), who showed that ATP inhibited the rabbit muscle pyruvate kinase reaction competitively with respect to both PEP* and ADP. Supporting this, they found ATP to interfere with

* Abbreviations: PEP, phosphoenolpyruvate; TMACl, trimethylammonium chloride.

the binding of both substrates to the enzyme. The K_m for one substrate was unaffected by the concentration of the other substrate, and Reynard *et al.* (1961) concluded that ADP and ATP share one binding site and that PEP and pyruvate share another binding site on the enzyme molecule. Thereby the inhibition by ATP was competitive with respect to ADP because of the sharing of the same binding site, and competitive with respect to PEP because of the common locus for their transferable phosphoryl group. The importance of the ATP inhibition for a closer understanding of the

mechanism of the pyruvate kinase reaction has been emphasized by Boyer (1962).

Lowry & Passonneau (1964) demonstrated ATP inhibition of brain pyruvate kinase, but this inhibition was found to be non-competitive with respect to either substrate. Yeast pyruvate kinase was also inhibited by ATP, but, since ITP, CTP, UTP, NADP⁺ and citrate also caused inhibition, this was regarded as non-specific (Hess, Haeckel & Brand, 1966). Mildvan & Cohn (1966) demonstrated ATP inhibition of the Mn²⁺-activated pyruvate kinase reaction that was competitive with respect to PEP. ATP inhibition has also been demonstrated for pyruvate kinase from muscle and liver (Tanaka, Harano, Sue & Morimura, 1967) and from adipose tissue, the last-named being competitive with PEP (Pogson, 1968).

McQuate & Utter (1959) have shown ATP inhibition of the reversed pyruvate kinase reaction, and the possible role of ATP inhibition of both the forward and the backward reaction for regulation of glycolysis and gluconeogenesis has been discussed (Krebs & Eggleston, 1965; Krebs, 1965). Inhibition by ATP has been introduced as an important factor in the computer simulation studies on the pyruvate kinase reaction by Kerson, Garfinkel & Mildvan (1967).

The role of both a univalent and a bivalent cation in the pyruvate kinase reaction has been extensively studied with various techniques (Kachmar & Boyer, 1953; Suelter & Melander, 1963; Kayne & Suelter, 1965; Sorger, Ford & Evans, 1965; Suelter *et al.* 1966). Melchior (1965) obtained results strongly suggesting that Mg²⁺ is required to form the MgADP⁻ complex, which was regarded as the specific substrate in the reaction, whereas K⁺ functions to activate the enzyme. The chelating ability of the adenine nucleotides has been extensively reviewed by Phillips (1966), and, considering the dependence on different methods and background electrolytes for determining the complex-formation constants, the mean constants for formation of MgADP⁻ and MgATP²⁻ are about $2.5 \times 10^3 \text{ M}^{-1}$ and $3.1 \times 10^4 \text{ M}^{-1}$ respectively at pH 7.5–8.8. Accordingly, the total magnesium concentration has to be 15 times that of total ADP to have 99% of the latter as MgADP⁻, and ATP will remove Mg²⁺ from this complex. (In this paper 'magnesium' refers to total Mg²⁺, both free and in the form of complexes.) Close examination of the experimental conditions used by those who have demonstrated ATP inhibition of pyruvate kinase reveals that this inhibition could partly be explained by the chelating effect of ATP. For example, after introduction of 7.5 mM-ATP into a system containing 2 mM-ADP and 10 mM-magnesium, only 1.6 mM-MgADP⁻ should be present (Reynard *et al.* 1961). In the system used

by Mildvan & Cohn (1966) 2.5 mM-ATP produced inhibition at 2 mM-ADP and 4 mM-manganese (total). However, under these conditions the MnADP⁻ concentration should be decreased by 25–30%, and this could, as noted by the authors, partly explain the inhibition. Similarly, complex-formation must be the main reason for the inhibition showed by Hess *et al.* (1966) by using 3 mM-, 8 mM- and 16 mM-ATP with 5 mM-magnesium and 1 mM-ADP.

The apparent importance of chelation in ATP inhibition of the pyruvate kinase reaction was emphasized by preliminary experiments in our laboratory showing that 6.8 mM-ATP had no effect on the reaction rate at 44 mM-magnesium at ADP concentrations up to 1 mM. The kinetics of the pyruvate kinase reaction at relatively high concentrations of the bivalent cation activator appear not to have been investigated. The present paper attempts to correlate the inhibition of the pyruvate kinase reaction by ATP, PP_i and EDTA with the concentration of magnesium. The concentrations of ADP, PEP and ATP in our experiments were similar to those used by Reynard *et al.* (1961). Their incubation system containing 100 mM-K⁺ and 10 mM-glycylglycine was also employed in addition to our usual system with 0.175 M-tris-hydrochloric acid and 56 mM-K⁺. The pH of both systems was 8.5.

MATERIALS

Buffers

Tris-HCl buffer, pH 8.5 (0.2 M), contained 0.28 M-mole of HCl/m-mole of tris (Trizma; Sigma Chemical Co., St Louis, Mo., U.S.A.) and had thus an apparent ionic strength of 0.056. The pH was not changed by fourfold dilution with water. A Radiometer model 27 pH-meter was used.

Glycylglycine-NaOH buffer, pH 8.5 (0.1 M), contained 0.64 M-mole of NaOH/m-mole of glycylglycine (Sigma Chemical Co.).

Chemicals

ADP. ADP (sodium salt) (from equine muscle; Sigma Chemical Co.) and ADP (sodium salt) (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) were stored at -25° and dissolved in water or 0.2 M-tris-HCl buffer, pH 8.5. The presence of ADP (2 mM or less) did not alter the pH of the buffer as did ATP (see below). The aqueous solutions of pH 3.8–4.0 were adjusted to pH 7.0 with 1.8 M-mole of KOH/m-mole of ADP. The exact concentration of ADP, determined as described below, varied between 75 and 95% of the stated value. The ADP solutions (0.2–2 mM) could be stored at -25° without any demonstrable change in concentration for 6 months. The preparations contained less than 0.012 M-mole of magnesium/m-mole of ADP.

ATP. ATP (crystalline disodium salt) (from equine muscle; Sigma Chemical Co.) was stored at -25° and dissolved in water or 0.2 M-tris-HCl buffer, pH 8.5. Ionization of ATP (16 mM) in the buffer lowered the pH to

8.1, which was readjusted to 8.5 with 1.4 m-moles of KOH/m-mole of ATP. Aqueous solutions of ATP (16 mM), which were pH 3.4, were adjusted to pH 7.0 with 1.2 m-moles of KOH/m-mole of ATP. The exact concentration of ATP, determined as described below, was 97–98% of the stated value. The ATP preparations were contaminated with 1.5–3% of ADP, the exact concentration of which was determined for every ATP solution prepared. Solutions of ATP (1–16 mM) could be stored at -25° without change in concentration for 6 months. The preparations contained less than 0.0035 m-mole of magnesium/mole of ATP.

NADH and NADP⁺. β -NADH (sodium salt) and β -NADP⁺ (sodium salt) (Sigma Chemical Co.) were stored at -25° . Solutions of 14 mM- β -NADH and 20 mM- β -NADP⁺ in 0.12 M-NaHCO₃ could be stored at 4° without demonstrable change in concentration for 1 month.

PEP. PEP (crystalline triethylammonium salt) (Sigma Chemical Co.) was stored at -25° . Solutions of 4–10 mM-PEP in 1.3 M-KCl or KCl-MgSO₄ mixtures of various compositions could be stored at 4° without change in concentration for 2 months. The concentration of PEP, determined as described below, was 97% of the stated value, and up to 2.5% of pyruvate contaminated the preparations.

Pyruvic acid. Pyruvic acid (crystalline sodium salt) (type II; Sigma Chemical Co.) was stored at 4° . Aqueous solutions could be stored at -25° without loss in concentration for 1 year. The concentration of pyruvate, determined as described below, was in exact accordance with the stated value.

Other chemicals. All other chemicals, except for TMACI from Fluka A.-G. (Buchs, Switzerland), were products (p.a.) of E. Merck A.-G. (Darmstadt, Germany). MgSO₄·7H₂O was used and the magnesium concentration, determined as described below, was in accordance with the stated value. PP₁ (tetrasodium salt) and EDTA (disodium salt) did not change the pH of the 0.2 M-tris-HCl buffer, pH 8.5, when dissolved to a concentration of 16 mM.

Enzymes

All suspensions of crystalline enzymes in (NH₄)₂SO₄ solution were kept at 4° .

Pyruvate kinase (EC 2.7.1.40). Pyruvate kinase (type II; crystalline from rabbit skeletal muscle; Sigma Chemical Co.), a suspension (10 mg./ml.) in (NH₄)₂SO₄ solution, was used. In all kinetic experiments a single preparation was employed. This was diluted with ice-cold water immediately before use and the diluted solution was kept at 0° during the experiment. The activity of the concentrated pyruvate kinase decreased by 32% during storage for 1 year. Its activity when received from the suppliers was 26 μ moles of pyruvate formed/min./mg. of enzyme (0.175 M-tris-HCl buffer, pH 8.5, 1.0 mM-ADP, 0.08 mM-PEP, 56.5 mM-KCl and 44 mM-MgSO₄ at 26°).

For the determination of ADP and PEP, pyruvate kinase from both Sigma Chemical Co. and C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), the latter being a suspension (10 mg./ml.) of crystalline enzyme in (NH₄)₂SO₄ solution, were used.

Lactate dehydrogenase (EC 1.1.1.27). Lactate dehydrogenase (type II; crystalline from rabbit muscle; Sigma Chemical Co.), a suspension (10 mg./ml. and 400 units/mg.) in (NH₄)₂SO₄ solution, and lactate dehydrogenase (crystal-

line from rabbit muscle; C. F. Boehringer und Soehne G.m.b.H.), as a suspension (5 mg./ml.) in 3.2 M-(NH₄)₂SO₄, were used.

Hexokinase (EC 2.7.1.1). Hexokinase (type C; crystalline from yeast; Sigma Chemical Co.), a suspension (1300 units/ml.) in 3.2 M-(NH₄)₂SO₄, was used.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Glucose 6-phosphate dehydrogenase (type VI; from yeast; Sigma Chemical Co.), a suspension (2 mg./ml. and 150 units/mg.) in (NH₄)₂SO₄ solution, was used.

METHODS

Determinations

All enzymic photometric determinations below were performed with a Beckman DK-2A ratio recording spectrophotometer, and ϵ 6220 at 340 nm. was used for both β -NADH and β -NADPH.

Determination of ADP. This was based on photometric measurement of β -NADH consumed when a pyruvate kinase system with excess of PEP was coupled to a lactate dehydrogenase system.

To 2 ml. of ADP (5–30 μ M) in 0.2 M-tris-HCl buffer, pH 8.5, in a quartz cuvette was added 100 μ l. of 8.5 mM-PEP in 1.3 M-KCl–0.4 M-MgSO₄ and 25 μ l. of 14 mM- β -NADH in 0.12 M-NaHCO₃. Lactate dehydrogenase (stock solution diluted 1:2 with water) was added by means of a plastic rod and the transmittance at 340 nm. was recorded against 0.095 mM- β -NADH (Holmsen & Stormorken, 1965). When the transmittance was constant (T_1), 10 μ l. of pyruvate kinase (stock solution diluted 1:2 with water) was injected into the sample cuvette. The transmittance rose rapidly to a constant value (T_2). Samples of the solvent for ADP that were treated in the same way gave $\log T_2 - \log T_1$ ranging from 0.002 to 0.006 (reagent blank). When corrected for this blank value the $\log T_2 - \log T_1$ values for the ADP solutions were directly proportional to the relative amount of ADP.

Determination of PEP. This was based on the same principles as for ADP, except that here ADP was in excess.

A 100 μ l. portion of PEP (0.2–1.0 mM) in 1.3 M-KCl–1 M-MgSO₄ was added to a cuvette containing 2 ml. of 1.9 mM-ADP in 0.2 M-tris-HCl buffer, pH 8.5, followed by 25 μ l. of 14 mM- β -NADH in 0.12 M-NaHCO₃. Further procedure was as described for ADP above.

Determination of ATP. This was based on photometric determination of β -NADPH formed when a hexokinase system with excess of glucose was coupled to a glucose 6-phosphate dehydrogenase system.

To 2 ml. of ATP (5–30 μ M) in 0.2 M-tris-HCl buffer, pH 8.5, in a cuvette was added 100 μ l. of 16 mM-glucose in 0.4 M-MgSO₄ and 50 μ l. of 20 mM- β -NADP⁺ in 0.12 M-NaHCO₃. Glucose 6-phosphate dehydrogenase (undiluted) was added by means of a plastic rod and the transmittance at 340 nm. was recorded against water. When the transmittance was constant (T_1), 10 μ l. of hexokinase (undiluted) was injected and the transmittance fell to a constant value (T_2). The $\log T_2 - \log T_1$ values for the ATP solutions were, when corrected for the reagent-blank value (0.002–0.006), directly proportional to the relative amount of ATP.

Determination of pyruvate. This was based on photometric estimation of β -NADH consumed in a lactate dehydrogenase system with excess of β -NADH.

A 2 ml. portion of pyruvate (10–40 μM) and 25 μl . of 7 mm- β -NADH in 0.12 M- NaHCO_3 were mixed in a cuvette. When the extinction at 340 nm., recorded against water, had reached a constant value (E_1), 10 μl . of lactate dehydrogenase (diluted 1:4 with water) was injected and the extinction fell to a constant value (E_2). The $E_1 - E_2$ values were, when corrected for the reagent-blank values, directly proportional to the pyruvate concentration.

Determination of magnesium. With incubation mixtures and MgSO_4 solutions this was performed by titration with 5 mm-EDTA of solutions (containing less than 20 mm-magnesium) buffered to pH 10.0 with 0.02 M-ethanolamine-0.01 M-HCl (final buffer concentration). The end point was determined visually with Eriochrome Black T.

With ADP and ATP solutions magnesium was determined by atomic absorption with a Unicam SP.90 atomic absorption spectrometer.

Kinetic studies

Determination of the initial velocity of the pyruvate kinase reaction was based on measurement of the amount of pyruvate liberated from PEP during incubation for a noted time.

Into plastic tubes were pipetted the components of either incubation mixture A or incubation mixture B. Incubation mixture A was prepared by pipetting in the following solutions: (a) 1.0 ml. of ADP (0.6–2.3 mm) in 0.2 M-tris-HCl buffer, pH 8.5; (b) 1.0 ml. of 0.2 M-tris-HCl buffer, pH 8.5, alone or containing ATP (5–16 mm), PP_i (16 mm), EDTA (16 mm) or TMACl (up to 563 mm); only the ATP solutions had to be readjusted to pH 8.5 with KOH; (c) 100 μl . of PEP (0.9–8.5 mm) in a mixture containing 1.3 M-KCl and various concentrations of MgSO_4 . Incubation mixture B, representing that of Reynard *et al.* (1961), was prepared by pipetting in the following solutions: (a) 0.5 ml. of ADP (0.97–3.9 mm) in water, neutralized with KOH; (b) 1.0 ml. of water or ATP (5–16 mm) in water, neutralized with KOH; (c) 0.37 ml. of PEP (0.51–2.21 mm) in a mixture containing 0.622 M-KCl and either 62 mm- or 273 mm- MgSO_4 ; (d) 0.23 ml. of 0.1 M-glycylglycine-NaOH buffer, pH 8.5. These 2.1 ml. incubation mixtures were equilibrated at 25° and the reaction was started with the addition of 200 μl . of pyruvate kinase solution. The reaction was stopped after a noted time of incubation by transferring 2.0 ml. of the incubation mixture to a tube containing 0.5 ml. of 2.5 M- HClO_4 at 0°. When all samples in an experiment had been brought to this step, 1.4 ml. of 1.2 M- K_2CO_3 in 1 M-tri(hydroxyethyl)ammonium chloride was added to each tube, causing immediate precipitation of KClO_4 . The crystals were removed by centrifugation at 3000 g_{max} . for 5 min. at –2°, and the supernatants were saved and kept frozen at –25° until pyruvate was determined, usually within 2 days.

During repetitions of particular experiments care was taken to alter the sequence in which the samples were processed.

The recovery of pyruvate was 100% during the procedure. ATP, ADP, KCl and MgSO_4 had no influence on the determination of pyruvate. Nor did PEP interfere when corrections for endogenous pyruvate were made.

The incubation time for a particular experiment was chosen within that period when pyruvate formation was linear with respect to time as well as to the pyruvate kinase

concentration. This usually meant reaction times of 1–2 min. The linearity was checked for each different composition of the incubation mixture. The concentration of pyruvate kinase and the incubation time were so inter-related that the pyruvate formed could be determined with a standard deviation of the mean not exceeding $\pm 5\%$. This corresponded to 17–67 μM -pyruvate formed in the incubation mixtures.

Controls

For each experiment controls were run to determine the endogenous pyruvate at zero time. Incubation mixtures were prepared as outlined above, except that ATP and ADP were replaced by their solvents. Instead of pyruvate kinase, 200 μl . of water was added, and the mixtures were treated as above. All experimental samples were corrected for the pyruvate found in these controls.

Calculations

The concentrations given for the substrates, ADP and PEP, as well as of ATP were arrived at by direct determinations. In mixtures of ADP and ATP account was taken of the amount of ADP present as contaminant in ATP, and the exact concentrations of ADP in such mixtures are given in the figures and tables.

K_m was obtained statistically by using Lineweaver-Burk plots and the method of the least squares. K_i was calculated by the procedure of Reynard *et al.* (1961) on the basis of ATP competition with two substrates as well as being calculated for competition with a single substrate.

RESULTS

Effect of ATP on the K_m for pyruvate kinase at high magnesium concentration. In the tris system (incubation mixture A) the K_m for ADP (9.8×10^{-4} M) was unaffected by ATP in concentrations up to 6.8 mm in the presence of 44 mm-magnesium (Fig. 1). Actually, high concentrations of ATP produced a small stimulation of the reaction rate. This phenomenon could be explained by an increase in the K^+ concentration due to the K^+ in the ATP preparations (1.4 m-mole/m-mole of ATP). Introduction of 6.8 mm-ATP thus raised the K^+ concentration from 56.5 to 66.0 mm, which corresponded to a 5% increase in the reaction rate (Table 1).

The K_m for ADP in the glycylglycine system (incubation mixture B) did not decrease with increasing amounts of ATP at 44 mm-magnesium (Fig. 2), as was found by Reynard *et al.* (1961) by using this system with 10 mm-magnesium. Here, neutral aqueous solutions of ATP and ADP were mixed and glycylglycine-sodium hydroxide buffer, pH 8.5, was added. However, the presence of 5.8 mm-ATP was found to cause a decrease in the final pH from 8.5 to 8.3. This was probably due both to the strong buffer capacity of the $\text{Na}^+ - \text{K}^+ - \text{ATP}$ solution (pH 7.0) and to the production of H^+

Fig. 1. Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.3 mM- (○), 4.5 mM- (Δ) and 6.8 mM- (□) ATP against the ADP concentration. The system contained 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 44 mM-MgSO₄, 56.5 mM-KCl and 0.87 μg. of pyruvate kinase/ml. The initial velocity, *v*, is expressed as μM-pyruvate/min.

Table 1. *Effect of K⁺ concentration on the velocity of the pyruvate kinase reaction*

The concentration of KCl was varied in incubation mixture A consisting of 1 mM-ADP, 0.08 mM-PEP, 44 mM-MgSO₄ and 0.87 μg. of pyruvate kinase/ml. The incubation mixtures were incubated at 25° and the amount of pyruvate produced in a noted time was determined.

Concn. of KCl (mM)	Reaction rate (μM-pyruvate/min.)
43.5	18.5
56.6	22.8
69.6	25.5
82.6	26.2

ions during the formation of Mg²⁺ complexes. A lowering of pH from 8.5 to 8.2 on the introduction of ATP was also observed with the tris system if the ATP dissolved in this buffer had not been adjusted to pH 8.5 in advance. At pH 8.2 the reaction rate was about 15% higher than at pH 8.5, indicating that the increase in reaction velocity when ATP was introduced in the glycylglycine system (Fig. 2)

Fig. 2. Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 1.9 mM- (○), 3.9 mM- (Δ) and 5.8 mM- (□) ATP against the ADP concentration. The system contained 0.01 M-glycylglycine-NaOH buffer, pH 8.5, 0.09 mM-PEP, 44 mM-MgSO₄, 100 mM-KCl and 0.87 μg. of pyruvate kinase/ml. The initial velocity, *v*, is expressed as μM-pyruvate/min.

was due both to increase in the K⁺ concentration and decrease in pH.

The *K_m* for ADP was markedly lower in the glycylglycine system (3.3×10^{-4} M) than in the tris system (9.8×10^{-4} M). This difference was apparently not related to the buffer concentration (0.175 M-tris-hydrochloric acid and 0.01 M-glycylglycine-sodium hydroxide) as dilution of the tris-hydrochloric acid buffer to 0.044 M did not alter the *K_m*, although the maximal velocity increased by 50%.

The *K_m* for PEP was unaffected by ATP in the presence of 44 mM-magnesium with both incubation systems (Figs. 3a and 3b). An increase in K⁺ concentration and fall in the pH in the glycylglycine system with increase of the ATP concentration were noted and probably contributed to the increase in the reaction rate as discussed above. However, the reaction rate was also raised by the increase in ADP concentration on the introduction of ATP (0.15 mM per 6.8 mM-ATP) and this has not been corrected for.

In the glycylglycine system the *K_m* for PEP (1.4×10^{-4} M) was lower than in the tris system (10.4×10^{-4} M). A comparison between the *K_m* values for ADP and PEP at 44 mM-magnesium shows that they equal each other in the tris system, whereas the *K_m* for ADP is about twice that of PEP in the glycylglycine system. Both *K_m* values were markedly higher in the tris system than in the glycylglycine system, a feature probably related to the difference in K⁺ concentration (56.5 and 100 mM respectively) as well as to the type of buffer.

Effect of the concentration of one substrate on the K_m for the other substrate at 44 mM-magnesium. The

Fig. 3. (a) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.3 mM- (○), 4.5 mM- (△) and 6.8 mM- (□) ATP against the PEP concentration. The system contained 0.175 M-tris-HCl buffer, pH 8.5, 1.8 mM-ADP, 44 mM-MgSO₄, 56.5 mM-KCl and 0.44 μg. of pyruvate kinase/ml. (b) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.1 mM- (○), 4.2 mM- (△) and 6.3 mM- (□) ATP against the PEP concentration. The system contained 0.01 M-glycylglycine-NaOH buffer, pH 8.5, 1.8 mM-ADP, 44 mM-MgSO₄, 100 mM-KCl and 0.87 μg. of pyruvate kinase/ml. In both (a) and (b) the initial velocity, *v*, is expressed as μM-pyruvate/min.

finding by Reynard *et al.* (1961) with rabbit skeletal-muscle pyruvate kinase at 10 mM-magnesium, that K_m for one substrate is unaffected by the concentration of the other substrate, has been shown to be a feature shared by the erythrocyte enzyme but not by the leucocyte enzyme (Campos, Koler & Bigley, 1965). Such a general change in the kinetic properties of the enzyme might also have been caused by high concentrations of magnesium, which greatly depresses the reaction rate (see below). The lack of ATP inhibition could thus be a result of such a change. Table 2 demonstrates, however, that the K_m for one substrate is independent of the concentration of the other substrate at 44 mM-magnesium with our skeletal-muscle pyruvate kinase preparation.

Inhibition by ATP of the pyruvate kinase reaction at 10 mM-magnesium. In the tris system ATP caused competitive inhibition with respect to ADP (Fig. 4a) with K_i 24×10^{-4} M as calculated for a single substrate. Our data, however, did not fit the rate equation given by Reynard *et al.* (1961) based on competition with both ADP and PEP. So calculated, the K_i values obtained were negative. ATP also caused inhibition in the glycylglycine system (Fig. 4b), but this did not appear strictly competitive as the K_i for single-substrate competition varied with the ATP concentration. Low amounts of ATP increased the affinity between

Table 2. Effect on K_m for one substrate of the concentration of the other substrate

Incubation mixtures containing 44 mM-MgSO₄, 56.6 mM-KCl, 0.046–0.184 mM-PEP, 0.46–1.83 mM-ADP, 0.175 M-tris-HCl buffer, pH 8.5, and the concentrations of pyruvate kinase indicated were incubated at 25° and the reaction was stopped with HClO₄ after noted times. The pyruvate content was measured and K_m values were determined.

Concn. of pyruvate kinase ...	0.87 μg./ml.		1.74 μg./ml.	
	Concn. of PEP (mM)	$10^4 K_m$ for ADP (M)	Concn. of ADP (mM)	$10^4 K_m$ for PEP (M)
	0.046	8.0	0.46	4.1
	0.092	11.2	0.92	4.7
	0.184	7.7	1.83	3.5

enzyme and ADP (lowering of the K_m ; the results obtained did not obey the criteria for competitive inhibition), whereas higher ATP concentrations slightly decreased this affinity with the K_i varying between 124×10^{-4} and 188×10^{-4} M. The inhibition by ATP in the glycylglycine system did not fit the equation for competition with both substrates, the K_i values being always negative.

ATP also gave competitive inhibition at 10 mM-magnesium with respect to PEP in the tris system

Fig. 4. (a) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.1 mM- (○), 4.2 mM- (△) and 6.3 mM- (□) ATP against the ADP concentration. The system contained 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 10 mM-MgSO₄, 56.5 mM-KCl and 0.87 μg . of pyruvate kinase/ml. (b) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.2 mM- (○), 4.3 mM- (△) and 6.3 mM- (□) ATP against the ADP concentration. The system contained 0.01 M-glycylglycine-NaOH buffer, pH 8.5, 0.09 mM-PEP, 10 mM-MgSO₄, 100 mM-KCl and 0.44 μg . of pyruvate kinase/ml. In both (a) and (b) the initial velocity, v , is expressed as μM -pyruvate/min.

Fig. 5. (a) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 2.3 mM- (○), 4.5 mM- (△) and 6.8 mM- (□) ATP against the PEP concentration. The system contained 0.175 M-tris-HCl buffer, pH 8.5, 1.9 mM-ADP, 10 mM-MgSO₄, 56.5 mM-KCl and 0.44 μg . of pyruvate kinase/ml. (b) Double-reciprocal plots of the velocity of the pyruvate kinase reaction at 0 mM- (●), 1.9 mM- (○), 3.9 mM- (△) and 5.8 mM- (□) ATP against the PEP concentration. The system contained 0.01 M-glycylglycine-NaOH buffer, pH 8.5, 1.8 mM-ADP, 10 mM-MgSO₄, 100 mM-KCl and 0.44 μg . of pyruvate kinase/ml. In both (a) and (b) the initial velocity, v , is expressed as μM -pyruvate/min.

(Fig. 5a). The K_i for single-substrate competition was $214 \times 10^{-4} \text{M}$, whereas that based on competition with both substrates was negative for the two lowest ATP concentrations used but equalled $2.57 \times 10^{-4} \text{M}$ for the highest ATP concentration. In the glycylglycine system ATP also inhibited the reaction, but did not appear to be strictly com-

petitive with respect to PEP as the K_i for single substrate decreased (62×10^{-4} – $17 \times 10^{-4} \text{M}$) with increasing ATP concentration (Fig. 5b). The K_i based on competition with both substrates also varied with the ATP concentration, being negative, $6.61 \times 10^{-4} \text{M}$ and $1.75 \times 10^{-4} \text{M}$ for 1.9 mM-, 3.9 mM- and 5.8 mM-ATP respectively.

The K_m values for the two substrates at 10mM-magnesium seemed to be dependent on the system used. For ADP they were 6.3×10^{-4} and 1.8×10^{-4} M whereas for PEP they were 1.8×10^{-4} and 0.6×10^{-4} M measured in the tris system and the glycylglycine system respectively. The higher K^+ concentration of the glycylglycine system (100mM) than in the tris system (56.5mM) might partly account for the difference, because the K_m for ADP decreased with increasing K^+ concentration in the tris system (Table 3). Irrespective of system, the K_m values for both PEP and ADP were distinctly lower at 10mM- than at 44mM-magnesium.

Effect on the reaction velocity of variation in the magnesium concentration in the absence and presence

Table 3. *Effect of K^+ concentration on the K_m for ADP*

Incubation mixtures containing 10mM- $MgSO_4$, 0.25–1.0mM-ADP, 0.09mM-PEP, 0.175M-tris-HCl buffer, pH 8.5, 0.87 μ g. of pyruvate kinase/ml. and various amounts of KCl were incubated at 25° for noted times, the reaction was stopped with $HClO_4$ and the amount of pyruvate was determined. The K_m values are based on Lineweaver-Burk plots treated by the method of the least squares.

Concn. of KCl (mM)	$10^4 K_m$ for ADP (M)
43.5	7.6
56.6	3.1
69.6	3.5
82.6	2.5

of ATP, PP_i or EDTA. Fig. 6(a) shows the effect of increasing magnesium concentration on the rate of the pyruvate kinase reaction in the absence and presence of 6.8mM-ATP in a tris system with ADP as rate-limiting factor (0.25mM-ADP, 0.09mM-PEP). With no ATP present the initial velocity increased with increasing magnesium concentration and reached a maximum at about 2mM, i.e. about eight to ten times the ADP concentration. Further increase in the magnesium concentration resulted in a decreasing reaction rate, and at 44mM it was only 25% of the maximal rate. When ATP was present, no pyruvate was formed until the magnesium concentration had reached 4–6mM. The velocity then increased and equalled that of the controls without ATP at 15–20mM-magnesium. Further increase in the magnesium concentration gave reaction rates slightly higher than those of the controls.

In Fig. 6(b) magnesium concentration was varied in a tris system with PEP as the rate-limiting factor (1.9mM-ADP, 0.09mM-PEP) in the absence and presence of 6.8mM-ATP. When ATP was absent, increase of the magnesium concentration caused the same variations in the reaction rate as the system with ADP as rate-limiting factor. In the presence of ATP the pyruvate kinase reaction was completely inhibited below 5mM-magnesium. The inhibition was abolished by a further increase in the magnesium concentration, but more slowly than when ADP was rate-limiting, which probably reflects the higher ADP/ATP concentration ratio

Fig. 6. (a) Effect of variation in the total magnesium concentration on the velocity of the pyruvate kinase reaction in the absence (●) and presence (□) of 6.8mM-ATP. The system contained 0.175M-tris-HCl buffer, pH 8.5, 0.09mM-PEP, 0.247mM- and 0.154mM-ADP in the absence and presence of ATP respectively, 56.5mM-KCl and 0.87 μ g. of pyruvate kinase/ml. The contaminating ADP in the ATP that was introduced led to an increase of 0.091mM-ADP in the incubation mixtures, thus giving the same final concentration of ADP in the presence and absence of ATP. (b) Effect of variation in the total magnesium concentration on the velocity of the pyruvate kinase reaction in the absence (●) and presence (□) of 6.8mM-ATP. The system contained 0.175M-tris-HCl buffer, pH 8.5, 1.9mM-ADP, 0.045mM-PEP, 56.5mM-KCl and 0.87 μ g. of pyruvate kinase/ml. In both (a) and (b) the initial velocity, v , is expressed as μ M-pyruvate/min.

in Fig. 6(b) than in Fig. 6(a). At 25mm-magnesium the reaction rate became equal to that of the controls without ATP and above this concentration the reaction velocities in the presence of ATP were slightly higher than in its absence.

In both systems (Figs. 6a and 6b) the maximal velocity of the pyruvate kinase reaction was markedly lower in the presence of ATP than in its absence. If the inhibition produced by ATP were only due to removal of Mg^{2+} from the $MgADP$ -complex by formation of the stronger $MgATP^{2-}$ -complex, the maximal reaction rate should be the same in the presence and absence of ATP. The only expected difference should be a displacement of the control reaction rates along the abscissae in Figs. 6(a) and 6(b) of about 6.8mm to the right. Hence ATP does produce an inhibition in both systems that is not due to chelation alone. As this inhibition is present at magnesium concentrations higher than that of ATP, where more than 99% of the nucleotide is in the $MgATP^{2-}$ form, it is most likely that this complex is the true inhibitor.

This could be shown experimentally by studying the effect of PP_i , ATP's chelating moiety (Phillips, 1966), on the pyruvate kinase reaction. This is shown in Fig. 7, where the magnesium concentration is varied in a tris system with ADP as rate-limiting factor in the absence and presence of 6.8mm-ATP or 6.8mm- PP_i (tetrasodium salt). The latter inhibited

the reaction completely at magnesium concentrations lower than 6.8mm, and further increase of the magnesium concentration gradually abolished the inhibition until a maximal reaction velocity was reached at about 16mm-magnesium that was only slightly lower than the maximal rate obtained in the absence of PP_i . In contrast, at above 10mm-magnesium all reaction rates in the presence of ATP (or $MgATP^{2-}$) were 25–30% lower than when PP_i was present, although these equalled that of the controls. At above 4mm-magnesium the presence of PP_i caused large precipitates, presumably of magnesium pyrophosphate, making part of the magnesium in the incubation mixture unavailable for the enzymic reaction. The abscissa in Fig. 7 expresses the total magnesium concentration, whereas the reaction in the presence of PP_i took place at lower magnesium concentrations. The reaction rates could be correlated to the true magnesium concentration, which was determined directly in the supernatant after removal by centrifugation of the insoluble material from the incubation mixtures. When so correlated the reaction rates at above 10–14mm-magnesium were the same in the samples containing PP_i as in the controls, as indicated by the open circles in Fig. 7.

In the presence of 6.8mm-EDTA in the system with ADP rate-limiting, no reaction took place at magnesium concentrations below 6–7mm (Fig. 8). Above this magnesium concentration the velocity

Fig. 7. Effect of variation in the total magnesium concentration on the velocity of the pyruvate kinase reaction in the absence (●) and presence of 6.8mm-ATP (□) or 6.8mm- PP_i (△). The system contained 0.175M-tris-HCl buffer, pH 8.5, 0.09mm-PEP, 0.247mm- and 0.154mm-ADP in the absence and presence of ATP respectively, or 0.247mm-ADP in both the absence and presence of PP_i , 56.5mm-KCl and 0.87 μ g. of pyruvate kinase/ml. Introduction of ATP led to an increase in the final ADP concentration of 0.091mm. The ○ symbols represent the concentration of magnesium after removal of insoluble $Mg_2P_2O_7$ (see the text). The initial velocity, v , is expressed as μ M-pyruvate/min.

Fig. 8. Effect of variation in the total magnesium concentration ($MgSO_4$) on the velocity of the pyruvate kinase reaction in the presence (▲) and absence (●) of 6.8mm-EDTA. The system contained 0.175M-tris-HCl buffer, pH 8.5, 0.09mm-PEP, 0.250mm-ADP, 56.5mm-KCl and 1.74 μ g. of pyruvate kinase/ml. The initial velocity, v , is expressed as μ M-pyruvate/min.

Fig. 9. Effect on the velocity of the pyruvate kinase reaction of increasing the ionic strength with MgSO_4 (●) and TMACl (○). The abscissa expresses the addition in ionic strength caused by these salts above that of the system, which consisted of 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 0.25 mM-ADP, 56.5 mM-KCl, 4 mM- MgSO_4 and 0.435 μg . of pyruvate kinase/ml. The initial velocity, v , is expressed as μm -pyruvate/min.

rose rapidly and reached a maximum at 10–12 mM-magnesium that was only 4–7% lower than the maximal value obtained with no EDTA present. Further increase of the magnesium concentration caused a decrease of the reaction rate that was similar to that of the control, but displaced to the right along the abscissa. In fact, the activity-magnesium concentration curves obtained with EDTA were basically those to be expected with chelation of Mg^{2+} as the only interference with the pyruvate kinase reaction. The only deviation was the slight lowering of the maximal activity and this might reflect the net increase in ionic strength (0.0136) by addition of EDTA (effect of ionic strength; cf. Fig. 9).

Effect of the pyruvate kinase reaction by variation of the ionic strength with magnesium sulphate or TMACl. The decrease in reaction velocity by increase of the magnesium concentration (Figs. 6–8) might reflect a specific inhibitory effect of the free Mg^{2+} ion as well as a general effect of the corresponding increase in the ionic strength, or it might be dependent on both effects. In Fig. 9 the ionic strength in a tris system with ADP as rate-limiting factor and 4 mM-magnesium has been increased by addition of TMACl or more magnesium sulphate. The values on the abscissa express the increment in ionic strength caused by the additions above that

of the system with 4 mM-magnesium (I 0.122). An increase in ionic strength of 0.160 (corresponding to an increase from 4 mM- to 44 mM-magnesium sulphate) produced 50% inhibition of the reaction velocity irrespective of the salt used. The reaction velocity decreased immediately on addition of TMACl , which was to be expected since the trimethylammonium ion is known not to interfere with the activation of the reaction by univalent cations (Melchior, 1965; Kayne & Suelter, 1965; Suelter *et al.* 1966; Wilson, Evans & Becker, 1967). On the other hand, Mg^{2+} is a specific activator of the process, and, as activation must precede any inhibition by increase of the magnesium concentration, the inhibitory phase should be displaced to higher ionic strengths than with the inert TMACl . This follows from Fig. 9, and after the reaction velocity had started to decrease with rising magnesium sulphate concentration it decreased in manner similar to that when TMACl was used. This indicates that the main part of the magnesium-induced inhibition was caused by the general effect on pyruvate kinase of increasing ionic strength, Mg^{2+} having little, if any, specific effect. It should be noted that the values for ionic strength in Fig. 9 are overestimated, since they are based on 100% dissociation of the electrolytes. However, there is good reason to believe that TMACl and magnesium sulphate are dissociated to the same extent in the concentrations used, and this is important for the above conclusion.

Inhibition of the pyruvate kinase reaction at 44 mM-magnesium by ATP with correction for its chelating effect. According to the equation:

additions of EDTA leads to an increase of the ionic strength of the systems that equals the increment in the concentrations of the Na^+ and H^+ ions. It therefore seems contradictory that the reaction rate actually increases on addition of EDTA to a system with high magnesium concentration (Fig. 8) when the reaction rate decreases with increasing ionic strength (Fig. 9). Nevertheless, with EDTA or another chelator, the reaction maximum is shifted to higher magnesium concentrations, and the relation between reaction rate and magnesium concentration is a compromise between activation and the inhibitory effect of high ionic strength. It follows that addition of a chelator at high magnesium concentrations causes an increase in reaction velocity because of the shift of the reaction maximum to higher magnesium concentrations. The effect of the corresponding increase in ionic strength is, as mentioned above, to lower the maximal reaction rate.

ATP is a chelator and thus shifts the maximal

rate of the pyruvate kinase reaction to higher magnesium concentrations. Reaction rates obtained with ATP must be corrected for this effect to

Fig. 10. Effect of variation in the total magnesium concentration (MgSO_4) on the velocity of the pyruvate kinase reaction in the presence of 6.8 mM-EDTA (▲) or 6.8 mM-ATP (□). The system contained 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 0.250 mM- and 0.203 mM-ADP in the absence and presence of ATP respectively, 56.5 mM-KCl and 1.74 μg . of pyruvate kinase/ml. Addition of ATP led to an increase in the ADP concentration of 0.047 mM. The initial velocity, v , is expressed as $\mu\text{M-pyruvate/min}$.

Fig. 11. Double-reciprocal plot of the velocity of the pyruvate kinase reaction against the ADP concentration in the presence of 6.8 mM-EDTA (▲) or 6.8 mM-ATP (□). The system contained 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 56.5 mM-KCl, 44 mM- MgSO_4 and 0.87 μg . of pyruvate kinase/ml. The initial velocity, v , is expressed as $\mu\text{M-pyruvate/min}$.

determine the inhibition due to ATP as such. One could, for example, compare the reaction rates in presence of ATP with those obtained without ATP and correspondingly less magnesium, or simply add Mg^{2+} plus ATP. But these methods result in relatively great differences in ionic strength between samples and controls. It seems better to use controls with the same composition as the experimental samples, but containing a chelating agent in the same concentration as ATP. Since EDTA only affects the pyruvate kinase reaction through chelation (Fig. 8), this chelator has been used in the controls by repetitions of the experiments recorded in Figs. 1, 3, 6(a) and 6(b).

In Fig. 10 the effect of 6.8 mM-ATP in the system with ADP as rate-limiting factor is compared with controls containing the same concentration of EDTA. At all magnesium concentrations where pyruvate was formed, i.e. above 5–7 mM, the samples containing ATP had lower reaction rates than the controls containing EDTA. However, the degree of ATP inhibition was not constant in the 7–44 mM-magnesium concentration range, but decreased with increasing magnesium concentration and was highest at the reaction maximum.

Reciprocal plots of reaction velocities against various ADP concentrations in the presence of 6.8 mM-ATP and 6.8 mM-EDTA at 44 mM-magnesium gave straight lines typical for competitive inhibition (Fig. 11). The degree of inhibition was small, the K_i for single-substrate competition being 1.2×10^{-2} M. It should be noted that this value is not corrected for the effect of increase in K^+ concentration introduced with ATP (about 10 mM). The K_m for ADP under these conditions was

Fig. 12. Effect of variation in the total magnesium concentration (MgSO_4) on the velocity of the pyruvate kinase reaction in the presence of 6.8 mM-EDTA (▲) or 6.8 mM-ATP (□). The system contained 0.175 M-tris-HCl buffer, pH 8.5, 0.09 mM-PEP, 1.84 mM-ADP, 56.5 mM-KCl and 1.74 μg . of pyruvate kinase/ml. The initial velocity, v , is expressed as $\mu\text{M-pyruvate/min}$.

Fig. 13. Double-reciprocal plot of the velocity of the pyruvate kinase reaction against the PEP concentration in the presence of 6.8 mM-EDTA (Δ) or 6.8 mM-ATP ($\square$). The system contained 0.175 M-tris-HCl buffer, pH 8.5, 1.84 mM-ADP, 56.5 mM-KCl, 44 mM-MgSO₄ and 0.35 μ g. of pyruvate kinase/ml. The initial velocity, v , is expressed as μ M-pyruvate/min.

7.9×10^{-4} M, whereas the results obtained did not fit the rate equation with ATP competition with both PEP and ADP (Reynard *et al.* 1961), as the K_i values were negative.

Fig. 12 shows the effect on the reaction velocity of variation in the magnesium concentration in a system rate-limited by PEP and containing 6.8 mM-ATP or 6.8 mM-EDTA. At above 6.8 mM-magnesium ATP caused progressively increasing inhibition when compared with the controls containing EDTA when the magnesium concentration was increased up to 44 mM. At this high magnesium concentration the ATP inhibition seemed to be competitive with PEP (Fig. 13) with a K_i for single-substrate competition equalling 3.31×10^{-2} M, whereas that based on competition with both substrates was negative.

DISCUSSION

ATP inhibits the pyruvate kinase reaction by two mechanisms. At magnesium concentrations lower than that of the nucleotide triphosphate the ATP⁴⁻ ion inhibits the reaction powerfully by removing Mg²⁺ from the substrate, MgADP⁻. This inhibition depends also on the ATP/ADP concentration ratio

because the complex-formation constant for MgATP²⁻ is about 12 times that of MgADP⁻. At magnesium concentrations higher than that of ATP, the presence of ATP causes a weak inhibition that is slightly dependent on the magnesium concentration (or on the ionic strength) and appears to be competitive with respect to both PEP and ADP.

This inhibition can hardly be explained by further removal of Mg²⁺ from MgADP⁻ by formation of bimetallic complexes of ATP (Lowenstein, 1958), since the formation constants for such complexes have been computed to 1×10^4 M⁻¹ for MgADP⁻ (Burton, 1959) and 2×10^4 – 7×10^4 M⁻¹ for MnADP⁻, ZnADP⁻ and CuADP⁻ (Handschin & Brintzinger, 1962), hence being 100 times less than that for MgADP⁻. As the complex-formation constant for MgATP²⁻ is about 1000 times that of Mg₂ATP (at 44 mM-magnesium the MgATP²⁻/Mg₂ATP concentration ratio is 226), the MgATP²⁻ complex is the predominating ATP species and probably the true inhibitor of the pyruvate kinase reaction.

The mechanism of the inhibition by MgATP²⁻ remains obscure. Our results do not conform to the rate equation given by Reynard *et al.* (1961), which is based on simultaneous competition between ATP and both ADP and PEP in a system with random addition of substrates and 'equilibrium' kinetics governing. The results obtained with the tris system always produced negative K_i values, whereas the values obtained with the glycylglycine system were either negative or decreased with increasing ATP concentration. Pogson (1968) has modified the equation of Reynard *et al.* (1961) in a way that corrects for the decrease in free Mg²⁺ by introduction of ATP. For the pyruvate kinase from adipose tissue he then obtained K_i values for an ATP inhibition being competitive with respect to PEP that were ten times those obtained by Reynard *et al.* (1961), but he could not demonstrate inhibition being competitive with respect to ADP. We have corrected for the interaction between ATP and Mg²⁺ experimentally by comparing the effects of ATP with those of the same amount of EDTA, but still our results do not fit the equation of Reynard *et al.* (1961). However, we have demonstrated competition between ATP and both ADP and PEP. This competition might occur separately with the two substrates, which requires calculation of K_i values for single-substrate competition. The K_i at 44 mM-magnesium for ATP versus PEP was found to be 170 times the K_m for this substrate and the K_i for ATP versus ADP and PEP indicates that MgATP²⁻ can hardly compete simultaneously with ADP and PEP for their binding to two different sites on the enzyme, as proposed by Reynard *et al.* (1961). It therefore seems unlikely that these

binding sites are located so close to each other as to facilitate the direct transfer of the phosphoryl group from bound PEP to bound ADP. The lack of strong MgATP^{2-} inhibition with K_i values of the same order as the K_m values and with K_i versus ADP equalling that versus PEP, as found by Reynard *et al.* (1961), does not preclude their suggestion about two different binding sites for ADP and PEP. Our pyruvate kinase preparation also showed the property of the K_m for one substrate being independent of the concentration of the other substrate at high magnesium concentrations, which is compatible with random combination of substrates and a two-site theory. Our data do not fit the two-site theory of Bücher (1947), suggesting one site for ATP and one for ADP in the 3-phosphoglycerate kinase reaction.

We were not able to reproduce the great inhibition by ATP of the pyruvate kinase reaction at 10mM-magnesium as reported by Reynard *et al.* (1961) even when using their incubation system. Some minor differences exist between our performance of the experiments and theirs, such as their reaction temperature being 0° whereas ours was 25°, and they terminated the reaction with 2,4-dinitrophenylhydrazine and measured the pyruvate formed colorimetrically as the dinitrophenylhydrazone formed after alkalization, whereas we stopped the reaction with perchloric acid and measured the pyruvate as the amount of β -NADH consumed in the lactate dehydrogenase reaction. Further, they did not apparently correct for the ADP contamination of the ATP solutions, and do not mention any change in pH on addition of neutralized ATP solutions to the reaction mixtures. However, none of these minor differences explains the much smaller effects of ATP obtained in our experiments as compared with theirs. However, the K_m values for ADP and PEP of $1.8 \times 10^{-4}\text{M}$ and $6 \times 10^{-5}\text{M}$ respectively, which were obtained by us using the conditions of Reynard *et al.* (1961), are close to their values of $2.1 \times 10^{-4}\text{M}$ and $3.2 \times 10^{-5}\text{M}$. In spite of this agreement, the inhibition by ATP when tested in their system was found to be qualitatively different from theirs, by not being competitive with respect to ADP and PEP. With the tris system at 10mM-magnesium, in which the pH did not change on addition of ATP solutions adjusted to pH 8.5, the inhibition appeared to be competitive. At this magnesium concentration the ATP inhibition is stronger than at higher magnesium concentrations, but as mentioned in the introduction the inhibition at 10mM-magnesium is partly due to chelation and is not a pure MgATP^{2-} effect.

The K_m values for ADP and PEP were always higher in the tris system than in the glycylglycine system at the same magnesium concentration. As

the K_m for ADP decreased with increasing K^+ concentration, the higher concentration of this ion in the latter rather than the former system might explain this difference in K_m values. However, according to Kachmar & Boyer (1953) the K_m for PEP is independent of the K^+ concentration in the range used in the present work. The K_m values might therefore also depend on the qualitative composition of the buffer, since they did not vary with buffer concentration. The latter independence indicates that the increase of K_m by increasing magnesium concentration is not due to the corresponding increase in ionic strength, but rather to a specific effect of Mg^{2+} . These effects on K_m values for the different components of the medium warrant closer examination. At present, when the mechanisms underlying these effects are unknown, a K_m value should necessarily be reported with specific reference to the system used for its measurement.

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