

Thermodynamics of the Pyruvate Kinase Reaction and the Reversal of Glycolysis in Heart and Skeletal Muscle*

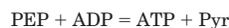
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Geoffrey P. Dobson^{‡§}, Sam Hitchins[‡], and Walter E. Teague, Jr.[¶]

From the [‡]Division of Physiology and Pharmacology, School of Biomedical and Molecular Sciences, James Cook University, Townsville, Queensland 4811, Australia and the [¶]Section of Nuclear Magnetic Resonance Studies, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Rockville, Maryland 20852

The effect of temperature, pH, and free $[Mg^{2+}]$ on the apparent equilibrium constant of pyruvate kinase (phosphoenol transphosphorylase) (EC 2.7.1.40) was investigated. The apparent equilibrium constant, K' , for the biochemical reaction P-enolpyruvate + ADP = ATP + Pyr was defined as $K' = [ATP][Pyr]/[ADP][P\text{-enolpyruvate}]$, where each reactant represents the sum of all the ionic and metal complexed species in M. The K' at pH 7.0, 1.0 mM free Mg^{2+} and I of 0.25 M was 3.89×10^4 ($n = 8$) at 25 °C. The standard apparent enthalpy (ΔH°) for the biochemical reaction was -4.31 kJmol^{-1} in the direction of ATP formation. The corresponding standard apparent entropy (ΔS°) was $+73.4 \text{ J K}^{-1} \text{ mol}^{-1}$. The ΔH° and ΔS° values for the reference reaction, $P\text{-enolpyruvate}^{3-} + ADP^{3-} + H^+ = ATP^{4-} + Pyr^{1-}$, were -6.43 kJmol^{-1} and $+180 \text{ J K}^{-1} \text{ mol}^{-1}$, respectively (5 to 38 °C). We examined further the mass action ratio in rat heart and skeletal muscle at rest and found that the pyruvate kinase reaction *in vivo* was close to equilibrium *i.e.* within a factor of about 3 to 6 of K' in the direction of ATP at the same pH, free $[Mg^{2+}]$, and T . We conclude that the pyruvate kinase reaction may be reversed under some conditions *in vivo*, a finding that challenges the long held dogma that the reaction is displaced far from equilibrium.

Pyruvate kinase (phosphoenol transphosphorylase) (EC 2.7.1.40) catalyzes the magnesium- and potassium-dependent transphosphorylation between phosphoenolpyruvate (P-enolpyruvate)¹ and ADP according to Reaction 1 (1).



REACTION 1

The enzymatic transfer of phosphate from P-enolpyruvate to ATP was first described in 1934 by Parnas, Ostern, and Mann (2). Despite early statements of Meyerhof *et al.* (3) that the reaction was irreversible, Lardy and Ziegler (4) experimentally showed in rat muscle extracts its reversibility in 1945 from the exchange of ³²P between P-enolpyruvate and ATP. These workers further showed that P-enolpyruvate could be synthesized directly from pyruvate in a system where ATP was constantly being regener-

ated and that the rate of reaction increased with increasing potassium concentration (4, 5). The reversibility of the reaction in muscle extracts was subsequently shown by Krinsky (6) and McQuate and Utter (7) in 1959 and again by Dyson *et al.* (8) in 1975.

The more important question, however, is whether pyruvate kinase can be reversed in intact tissues. Can glycolysis be reversed at pyruvate kinase and glycogen form from lactate in skeletal muscle *in vivo* (7, 9–14)? Because the early thermodynamic study of McQuate and Utter (7) indicated an unfavorable equilibrium at the pyruvate kinase step, Krebs proposed a bypass reaction via pyruvate carboxylase (EC 6.4.1.1). Pyruvate carboxylase catalyzes the ATP-driven formation of oxaloacetate from pyruvate and HCO_3^- and P-enolpyruvate carboxykinase (PEPCK) (EC 4.1.1.49), which then converts oxaloacetate to P-enolpyruvate (15). Although this pathway is known to occur in liver (and kidney) (16, 17), it remains unclear whether the enzymes are present in skeletal muscle or heart (18).

On closer examination of the otherwise excellent study of McQuate and Utter (7), there are a number of unavoidable limitations to their analysis. For example, in the 1950s there was considerable uncertainty about the apparent magnesium and acid binding constants for the ATP and ADP series, the binding of ADP in skeletal muscle, and the problem of measuring accurately low concentrations of metabolites in intact tissue. There was also a lack of computing power to solve the system of simultaneous equations defining the reaction *in vitro*. These limitations, along with the high sensitivity of the reaction to varying pH and free $[Mg^{2+}]$, have impeded a detailed study into the thermodynamics of the reaction. The aim of this study was to examine the thermodynamics of the pyruvate kinase reaction and estimate the apparent equilibrium position in heart and skeletal muscle using the methodologies described for our earlier work on creatine kinase and arginine kinase (19, 20). The present study provides strong evidence that the pyruvate kinase equilibrium may be reversed *in vivo*, which helps explain otherwise perplexing data on the reversal of glycolysis and glycogen synthesis following exercise.

MATERIALS AND METHODS

Enzymes and Chemicals—Pyruvate kinase (EC 2.7.1.40) from rabbit skeletal muscle was purchased as the 3.2 M ammonium sulfate suspensions from Roche Molecular Biochemicals. Pyruvate-monosodium salt, ATP-crystallized disodium salt, ADP-disodium salt, NADH-monosodium salt (grade 1) and NADP-disodium salt (98%), creatine kinase, glucose-6-phosphate dehydrogenase, hexokinase from yeast, and lactate dehydrogenase from beef heart were obtained from Roche Molecular Biochemicals. Pyruvate kinase (lyophilized powder from rabbit skeletal muscle), P-enolpyruvate-monopotassium salt, D-glucose, imidazole (low fluorescence blank 0.002%) and trizma-base (Tris[hydroxymethyl]-aminomethane), phosphate-potassium salt (monobasic and di-

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§ To whom correspondence should be addressed. Fax: 61-747-816279; E-mail: geoffrey.dobson@jcu.edu.au.

¹ The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase.

basic), and EDTA (acid form) and hydrogen peroxide 30% (9.8 M) were purchased from Sigma. All other chemicals were reagent grade.

Equilibrium Studies—The pyruvate kinase reaction was carried out from the forward and reverse direction in a reaction buffer containing 50 mM potassium phosphate, pH 7.0, 110 mM potassium chloride, and 4.75 mM magnesium chloride at 25 °C. For the forward direction, the reaction buffer also contained 0.5 mM ADP, 0.5 mM P-enolpyruvate, 4.3 mM ATP, and 4.7 mM pyruvate. For the reverse direction the reaction buffer also contained 5.0 mM pyruvate and 5.1 mM ATP. A 10-ml aliquot of each reaction buffer was placed in separate 10-ml conical bottom reaction vials with V-shaped magnetic stirring bars (Pierce). These reaction vials were sealed with removable Teflon caps and placed in a Neslab RTE100 temperature-controlled water bath. Experimental temperature was maintained ± 0.1 °C. A water/air-powered magnetic stir motor was used to mix the reaction vials throughout the experiment. Reaction buffer pH was monitored using a Radiometer Copenhagen PHM 93 reference pH meter, with a Radiometer Copenhagen PHC 2005 electrode. The procedure has been described in detail in Teague and Dobson (19, 20).

During the temperature experiments the pH meter was calibrated at each experimental temperature using the Radiometer Copenhagen 47.5 mM phosphate S11M004, pH 7.0 ± 0.01 , at 25 °C. This pH standard had a temperature coefficient ($\Delta\text{pH}/\Delta t$ °C) of -0.0028 , which was appropriate for our reaction buffers that contained 50 mM phosphate. The vials containing the reaction buffer were allowed 20 min to reach temperature prior to initiation of the reaction with enzyme. The pyruvate kinase enzyme was prepared by dissolving 10 mg of the lyophilized powder (5000 units per mg) in 1.0 ml of 50 mM phosphate buffer at pH 7.0, and 10 μl of this preparation was added to the 10 ml of reaction buffer to initiate the reaction (final activity of 5 units/ml at 25 °C). After 1 h of reaction time, a 1.0-ml aliquot was transferred from the reaction vial to a Centricon-30 spin filter (Amicon) maintained at the experimental temperature in the water bath. The spin filter was then placed into a temperature-controlled rotor and centrifuged at $4000 \times g$ for 5 min. The enzyme-free filtrate (~ 300 μl) containing the reaction buffer at equilibrium was removed and kept at -80 °C until analysis the following day. To ensure that the temperature was maintained at a constant throughout the centrifugation, great care was taken to keep the centrifuge and rotors at the appropriate experimental temperature. After completing the first experiment at 5 °C, the bath temperature was raised to 15 °C, and identical procedures were used for equilibration and sampling. The process was then repeated at 25, 35, and 45 °C. Experiments studying the variation of the K' and pH were carried out in a similar manner at 25 °C.

Measurement of ATP, Pyruvate, ADP, and P-enolpyruvate in Equilibrium Mixtures—Enzymatic assays of ATP and pyruvate were carried out according to the procedures described in Lowry and Passonneau (40) with the following modifications: ATP was measured spectrophotometrically in 50 mM Tris-HCl, pH 8.1, 0.4 mM D-glucose, 1 mM MgCl_2 , 0.3 mM NADP containing 0.35 units/ml glucose-6-phosphate dehydrogenase. The reaction was initiated with 0.7 units/ml hexokinase and complete in 5 to 10 min. Pyruvate was measured spectrophotometrically in 50 mM phosphate buffer, pH 7.0 (30 mM K_2HPO_4 , 20 mM KH_2PO_4), and 0.1 mM NADH. The reaction was initiated by the addition of 1.5 units/ml lactate dehydrogenase, and the reaction was complete in 5 to 10 min. P-enolpyruvate or ADP was measured by monitoring NADH fluorescence in the equilibrium samples after pyruvate had been removed by peroxide treatment. Pyruvate removal was essential, because high concentrations interfered with the accurate measurement of the micromolar concentrations of P-enolpyruvate and ADP, a consequence of the overall equilibrium strongly favoring pyruvate and ATP formation.

Pyruvate was removed by heating 100 μl of sample in 10×75 -mm tubes for 10 min at 60 °C in 50 mM imidazole, pH 7.5, and a 5:1 molar excess of H_2O_2 -pyruvate. Control experiments using standards showed that 99–100% of the pyruvate was removed by the peroxide oxidation step, without any effect on P-enolpyruvate or ADP concentrations; there was a 100% recovery of P-enolpyruvate or ADP standards. The excess peroxide was removed by the addition of 25 units/ml catalase (EC 1.11.1.6) to minimize reduction of NADH in subsequent enzymatic measurements but was found not to be necessary in our measurements. P-enolpyruvate or ADP was then measured by adding 1 ml of reagent and the pyruvate kinase-lactate dehydrogenase coupled assay system (21). Briefly, the reagent comprised a 50 mM phosphate buffer, pH 7.0 (30 mM Na_2HPO_4 , 20 mM NaH_2PO_4), 2 mM MgCl_2 , 0.2 mM ADP, 0.2 mM EDTA, 5 μM NADH, catalase (25 units/ml), and lactate dehydrogenase (50 μl of 10 mg/ml/50 ml). An initial reading was taken, after which 10 μl of pyruvate kinase (5 μl 10 mg/ml of water) was added to the reaction

in a volume of 10 μl (1.25 units) to measure the P-enolpyruvate. ADP could be measured by the substitution of 0.2 mM P-enolpyruvate for the ADP in the same reagent. The time for completion of either P-enolpyruvate or ADP measurement was 5 to 10 min. Standard curves were used for quantify the measurements.

Acid-dissociation and Magnesium Binding Constants at Varying Temperatures and $I = 0.25$ M—The acid-dissociation constants and magnesium binding constants for the ATP and ADP series, and for phosphate, and their respective ΔH° values (ionic strength = 0) used in this study can be found in Teague and Dobson (19) and Golding *et al.* (23). The acid-dissociation constants were of the form $\text{HA} = \text{H}^+ + \text{A}^-$ where $K_a = [\text{H}^+][\text{A}^-]/[\text{HA}]$, and magnesium binding constants were of the form $\text{Mg}^{2+} + \text{A}^x = \text{MgA}^{(2+x)}$ where $K_b = [\text{MgA}^{(2+x)}]/[\text{Mg}^{2+}][\text{A}^x]$ where HA is the acid, and A is its conjugate base. The method for adjusting the equilibrium constants to 5, 15, 25, 35, and 45 °C at the ionic strength of 0.25 M is also found in these papers. The K_a values using a ΔH° ($I = 0$) of -4.00 kJmol^{-1} for P-enolpyruvate were as follows: 5.019×10^{-7} (5 °C), 4.727×10^{-7} (15 °C), 4.4700×10^{-7} (25 °C), 4.242×10^{-7} (35 °C), and 4.039×10^{-7} (45 °C) (22). The K_b values using a ΔH° ($I = 0$) of $+12.50$ kJmol^{-1} for P-enolpyruvate were as follows: 1.253×10^2 (5 °C), 1.511×10^2 (15 °C), 1.800×10^2 (25 °C), 2.120×10^2 (35 °C), and 2.470×10^2 (45 °C) (22). Unfortunately we could not find a ΔH° ($I = 0$) for acid or magnesium binding to pyruvate and have used a K_a of 3.16×10^{-3} (5 to 45 °C) and a K_b of 6.3 (5 to 45 °C) (22). The K_b was assumed to be the same as the calcium binding constant. For such weak binding we would not expect a very large ΔH° ,² and thus it would have little impact on our results.

Calculation of Free $[\text{Mg}^{2+}]$ and the Concentration of Major Ionic Species in Equilibrium Mixtures—Free $[\text{Mg}^{2+}]$ refers to the concentration of ionized magnesium as opposed to magnesium bound to compounds such as ATP or other phosphates. Free $[\text{Mg}^{2+}]$, and the concentration of major ionic species of reactants of the pyruvate kinase reaction, were calculated using a computer program written in the language of *Mathematica*[®] (Wolfram Research). The details of the method and system of equations can be found in Teague and Dobson (19) and Golding *et al.* (23). Briefly, total concentrations of reactants of the reaction at equilibrium, together with the total magnesium concentration, ionic strength, and the acid-dissociation and metal binding constants adjusted to specified ionic conditions, were substituted into a system of simultaneous equations representing the pyruvate kinase reaction under our experimental conditions. The solution of these simultaneous equations yields molar concentrations for the ionic species of reactants present.

NMR Experiments: Determination of the Mass Action Ratio *in Situ*—To assess the equilibrium position of the biochemical reaction of pyruvate kinase (Reaction 1) in heart and skeletal muscle *in situ*, all the reactants, as well as pH and free $[\text{Mg}^{2+}]$, have to be measured or calculated in the tissues. From these data, the mass action ratio can be calculated and compared with the equilibrium constant determined in the laboratory after adjustment to the tissue pH, free $[\text{Mg}^{2+}]$ at 38 °C, and ionic strength of 0.25 M.

Fourteen male Sprague-Dawley rats weighing 300–350 g were obtained from the James Cook University breeding colony and housed in the animal facility. Seven were prepared for heart measurements and seven for muscle measurements. Rats were supplied with unrestricted access to food and water. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg rat weight⁻¹). The left femoral artery and vein were cannulated with PE 50 tubing, and the venous line was used for maintenance of anesthesia (15 mg/ml⁻¹ solution of sodium pentobarbital in 0.9% saline) whereas the arterial line was for blood collection and continuous monitoring of blood pressure. The details of the procedure are found in Hitchins *et al.* (24). Briefly, animals were artificially ventilated on room air with a Harvard small animal ventilator (Harvard Apparatus, South Natick, MA) to give a blood $p\text{O}_2$ of ~ 90 mm Hg, a $p\text{CO}_2$ of ~ 40 mm Hg, and a pH of ~ 7.4 . Blood (0.2 ml) was drawn from the arterial line and analyzed with the Ciba-Corning 865 blood gas analyzer (Ciba Corning Diagnostic Corp., Medfield, MA) to ensure that blood gases were within the correct range.

For heart measurements a thoracotomy was performed, and a custom-built, flexible arm surface coil (9-mm outer diameter) tunable to ³¹P was placed on the left ventricle. The coil was made of Teflon-coated copper wire (1.25-mm thick) and was sufficiently flexible to follow the movement of the heart and maintain contact, without excessive pressure against the heart (24). For skeletal muscle measurements, anesthesia was maintained by delivering 1% isoflurane (Abbott Australasia,

² R. Alberty, personal communication.

TABLE I
Attainment of equilibrium from the forward and reverse directions for the pyruvate kinase reaction at 25 °C

Average K'_{AK} forward direction = 37821 ± 628 (S.E.) ($n = 3$). Average K'_{AK} reverse direction = 37618 ± 1394 (S.E.) ($n = 3$). Percent difference = 1.0% (see "Results" for further discussion). K' is the apparent biochemical equilibrium constant at pH 7.0, free $[Mg^{2+}]$.

| Initial Concentrations | | | | Final Concentrations | | | | | | K'_{PKex} | K' (pH 7.0, free $[Mg^{2+}] = 1.0$ mM, $I = 0.25$ M) |
|---|-------|-------|-------|----------------------|--------|-------|-------|-------|----------|-------------|--|
| [ADP] | [PEP] | [ATP] | [Pyr] | [ADP] | [PEP] | [ATP] | [Pyr] | pH | Free[Mg] | | |
| 10^{-3} M | | | | 25 °C, 10^{-3} M | | | | | | | |
| Forward direction PEP + ADP = Pyr + ATP | | | | | | | | | | | |
| 0.50 | 0.50 | 4.30 | 4.70 | 0.133 | 0.0056 | 4.65 | 5.18 | 6.989 | 0.618 | 32016 | 36662 |
| 0.50 | 0.50 | 4.30 | 4.70 | 0.133 | 0.0054 | 4.67 | 5.21 | 6.980 | 0.616 | 33750 | 37986 |
| 0.50 | 0.50 | 4.30 | 4.70 | 0.126 | 0.0056 | 4.82 | 5.01 | 6.980 | 0.591 | 34057 | 38816 |
| Reverse direction Pyr + ATP = PEP + ADP | | | | | | | | | | | |
| 0 | 0 | 5.0 | 5.10 | 0.137 | 0.0050 | 4.84 | 5.11 | 6.940 | 0.557 | 36415 | 39216 |
| 0 | 0 | 5.0 | 5.10 | 0.134 | 0.0052 | 4.70 | 5.13 | 6.940 | 0.543 | 34439 | 34843 |
| 0 | 0 | 5.0 | 5.10 | 0.127 | 0.0054 | 4.79 | 5.04 | 6.950 | 0.564 | 35392 | 38795 |

Kurnell, Australia) in compressed air via the ventilator at a rate of 0.5 liter/min. Like in the heart protocol, the animal was transferred to a purpose-built Perspex cradle. The cradle was pre-fitted with a 37 °C water-heated pad. The gastrocnemius was exposed, and the muscle was carefully denuded of overlying tissue and covered with a plastic film to prevent drying of exposed tissue. A three-turn surface coil (14-mm outer diameter) tunable to ^{31}P was placed on the center of the gastrocnemius (25, 26).

^{31}P NMR experiments were performed at 121.47 MHz in a 110-mm horizontal bore Oxford 7.05-tesla superconducting magnet coupled to a Varian INOVA NMR spectrometer (24). For heart, radiofrequency pulses of 8- μ s duration at an approximate 40° flip angle were applied with a 1-s interpulse delay. FIDs were acquired over 0.4 s with a total of 1024 free induction delays averaged. An 8000-Hz spectral width was used, and 6400 data points were obtained. A 10-Hz exponential line-broadening factor was applied to ^{31}P NMR spectra, which were fitted using Varian Fitspec software. Following integration, all peaks were multiplied by a saturation correction factor specific for each peak. These factors were determined experimentally by comparing the peak integrals of partially relaxed spectra, obtained using the acquisition parameters described above, to the peak integrals of fully relaxed spectra (20-s interpulse delay). In heart, the mean correction factors for P_i , phosphocreatine, and β -ATP were 0.97 ± 0.08 , 1.24 ± 0.04 , and 0.87 ± 0.03 (\pm S.E., $n = 7$), respectively (24, 26). Upon completion of spectral acquisition, the heart was freeze-clamped at liquid nitrogen temperature. Tissue was ground to a powder in liquid nitrogen and stored at -80 °C for later enzymatic analysis of tissue ATP concentration and total creatine (24, 26).

For skeletal muscle, radiofrequency pulses of 8- μ s duration at an approximate 90° flip angle were applied with a 1-s interpulse delay (24, 26). FIDs were acquired over 0.8 s with a total of 256 FIDs averaged. A 6000-Hz spectral width was used, and 9600 data points were obtained. In contrast to heart spectra, a line-fitting program was not required for muscle because of the superior signal to noise ratio. The partially relaxed ^{31}P spectra were calibrated by comparison with fully relaxed spectra using the same process described for heart. In muscle, the mean correction factors for P_i , phosphocreatine, and β -ATP were 0.98 ± 0.10 , 1.03 ± 0.01 , and 0.88 ± 0.09 (\pm S.E., $n = 6$), respectively. Upon completion of spectral acquisition, the muscle was freeze-clamped, and the tissue was powdered in liquid nitrogen for enzymatic determination of ATP, total creatine, pyruvate, and P-enolpyruvate contents. The extraction procedures are described by Hitchins *et al.* (24).

Free [ADP] was calculated using the creatine kinase equilibria described in detail by Cieslar and Dobson (25, 26). To convert μ mol/g to mM intracellular water the spaces of Cieslar *et al.* (27) for rat heart and skeletal muscle were used. The ATP measured enzymatically was equated with the integral of the β -ATP peak for both heart and muscle spectra taking into account the saturation correction factors. The equations are described in detail in Hitchins *et al.* (24). Intracellular pH was calculated from the chemical shift (δ , ppm) of P_i relative to phosphocreatine in the ^{31}P spectra using the NMR version of the Henderson-Hasselbalch equation, shown below.

$$pH_i = 6.75 + \log\left(\frac{\delta - 3.25}{5.69 - \delta}\right) \quad (\text{Eq. 1})$$

Intracellular free $[Mg^{2+}]$ concentration ($[Mg^{2+}]_i$) was calculated from the observed chemical shift difference ($\delta_{\alpha\beta}$) in ppm between β -phosphate and α -phosphate resonances of ATP in the ^{31}P spectra using the modified form of the London equation (28), shown in Equation 2,

$$[Mg^{2+}]_i = K_D \left\{ \frac{\delta_{\alpha\beta}(1 + \alpha) - (\delta_1 + \alpha\delta_2)}{\delta_3 + \beta\delta_4 - \delta_{\alpha\beta}(1 + \beta)} \right\} \quad (\text{Eq. 2})$$

where $\alpha = [H^+]/K_H$ and $\beta = \alpha(K_D/K_D')$. K_H is the dissociation constant for the H/ATP^{4-} equilibrium, K_D is the dissociation constant for the ATP^{4-}/Mg^{2+} equilibrium, and K_D' is the dissociation constant for the $ATPH^3-/Mg^{2+}$ equilibrium. The parameters δ_1 , δ_2 , δ_3 , and δ_4 were assigned published values of 10.600, 11.660, 8.165, and 8.52 ppm, respectively, K_D was 9.0×10^{-5} M, K_H was 3.4×10^{-7} M, and K_D' was 7.2×10^{-4} M (24).

Statistical Significance—Values are reported as mean \pm S.E. Statistical significance was assessed using a student's t test or a two-way repeated measured analysis of variance (ANOVA). The α level of significance for all experiments was set at $p < 0.05$.

RESULTS

Attainment of equilibrium was judged complete when the apparent K' , defined as $[ATP][Pyr]/[ADP][P\text{-enolpyruvate}]$ agreed to within 10% when approached from both directions. The initial and final concentrations of reactants, experimental pH, and free $[Mg^{2+}]$ at 25° and the K' experimental (calculated at experimental conditions) and K' (calculated at specified pH 7, free $[Mg^{2+}]$ 1.0 mM, and $I = 0.25$ M) are shown in Table I. The average K' in the forward direction was 37,821, and in the reverse it was 37,618. The enthalpy (ΔH°) of the pyruvate kinase biochemical reaction in the ATP direction was -4.31 kJmol $^{-1}$ (Fig. 1) and calculated from the log K' versus temperature ($1/T$) where the slope of the line is equal to $-\Delta H^\circ/2.303 R$ (R is the gas constant 8.314 J mol $^{-1}K^{-1}$). The corresponding entropy (ΔS°) was +73.4 J mol $^{-1}K^{-1}$. The equation for the line in Fig. 1 was $y = 224.83X + 3.833$ ($R^2 = 0.97$).

A similar plot was constructed for the chemical reaction $P\text{-enolpyruvate}^{3-} + ADP^{3-} + H^+ = ATP^{4-} + Pyr^{1-}$, and the enthalpy and entropy values for the reaction were -6.43 kJmol $^{-1}$ and +190 J mol $^{-1}K^{-1}$, respectively. The equation of the line was $y = 335.99X + 9.934$ ($R^2 = 0.90$) (plot not shown). When the magnesium-bound reactants are used in the chemical reaction ($MgP\text{-enolpyruvate}^{1-} + MgADP^{1-} + H^+ = MgATP^{2-} + MgPyr^{1+}$), the enthalpy and entropy were -4.10 kJmol $^{-1}$ and +207 J mol $^{-1}K^{-1}$, respectively. The equation of the line was $y = 214.12X + 10.798$ ($R^2 = 0.88$) (plot not shown). The thermodynamic data for the different forms of the pyruvate kinase reaction, including the ΔG° and equilibrium constants, are summarized in Table II. The effect of pH on the pyruvate kinase equilibrium is shown in Fig. 2. As pH varied from 6.40 to 7.8 at free $[Mg^{2+}]$ of 1.0 mM, $I = 0.25$ M, and 25 °C, the K' for pyruvate kinase decreased by a factor of 10 (from around 100,000 to 10,000). It is also noteworthy that at lower pH the standard errors increased because of the very large equilibrium constants and decreasing micromolar concentrations of P-enolpyruvate and ADP.

The concentration of reactants of the pyruvate kinase reac-

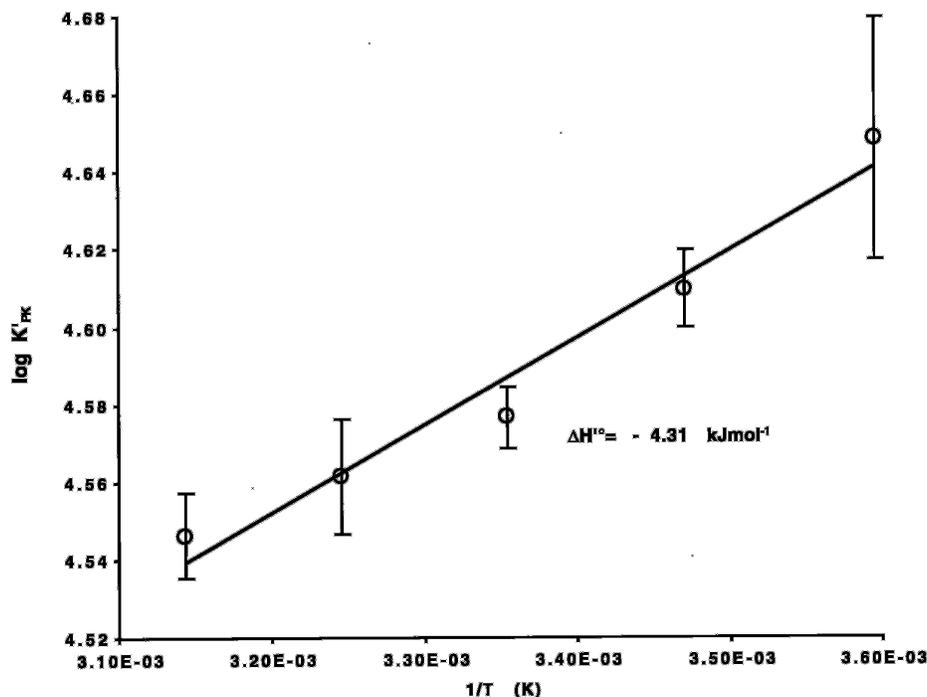


FIG. 1. Van't Hoff plot of the effect of temperature on the apparent equilibrium constant of the pyruvate kinase reaction (K'_{PK}). Each value represents the mean \pm S.E. ($n = 6$) at pH 7.0, free Mg^{2+} of 1.0 mM, and ionic strength of 0.25 M at 5, 15, 25, and 35 and 45 °C. The equation to the line was $y = 224.83X + 3.833$; $R^2 = 0.97$. The standard apparent heat of the reaction, $\Delta H'^{\circ}$, was calculated from the slope of the line (slope = $-\Delta H'^{\circ}/2.303 R$, where R is the gas constant $8.314 \text{ J mol}^{-1} \text{ K}^{-1}$). The intercept of $\log K'$ versus $1/T$ at $1/T = 0$ is equal to $\Delta S'^{\circ}/2.303 R$, where $\Delta S'^{\circ}$ is defined as the standard apparent entropy of the reaction under the conditions defined. The standard apparent $\Delta H'^{\circ}$ for the pyruvate kinase reaction in the direction of ATP formation was -4.31 kJmol^{-1} and $\Delta S'^{\circ} = +73.4 \text{ J K}^{-1}\text{mol}^{-1}$.

TABLE II
Thermodynamic properties of the pyruvate kinase reaction

All values are at $I = 0.25 \text{ M}$. The percentages in parentheses for enthalpy and T ΔS term are relative to the Gibbs energy.

| Biochemical reaction | K'_{PK} [Mg^{2+}] = 1.0 mM pH = 7.0 25 °C | $\Delta G'^{\circ}$ kJ mol $^{-1}$ 25 °C | $\Delta H'^{\circ}$ kJ mol $^{-1}$ 5–45 °C | $\Delta S'^{\circ}$ Jmol $^{-1}$ K $^{-1}$ 5–45 °C | T $\Delta S'^{\circ}$ kJ mol $^{-1}$ 25 °C |
|---|---|--|--|--|--|
| PEP + ADP \leftrightarrow Pyr + ATP | 3.89×10^4 | -26.2 | -4.31 (16%) | +73.4 | +21.9 (84%) |
| Chemical reactions | K'_{refPK} 25 °C | ΔG° kJmol $^{-1}$ 25 °C | ΔH° kJmol $^{-1}$ 5–45 °C | ΔS° Jmol $^{-1}$ K $^{-1}$ 5–45 °C | T ΔS° kJmol $^{-1}$ 25 °C |
| PEP $^{3-}$ + ADP $^{3-}$ + H $^{+}$ \leftrightarrow ATP $^{4-}$ + Pyr $^{-}$ | 1.09×10^{11} | -63.0 | -6.43 (10%) | +190 | +56.7 (90%) |
| MgPEP $^{1-}$ + MgADP $^{1-}$ + H $^{+}$ \leftrightarrow MgATP $^{2-}$ + MgPyr $^{+}$ | 3.24×10^{11} | -65.7 | -4.10 (6%) | +207 | +61.6 (94%) |

tion in rat heart and gastrocnemius skeletal muscle *in situ* are shown in Table III. The pH and free $[Mg^{2+}]$ were estimated from ^{31}P NMR and found to be 7.32 and 7.18 mM and 0.46 and 0.57 mM for heart and skeletal muscle, respectively (Table III). Free [ADP] was calculated from the creatine kinase equilibrium using the reactants reported in Table III and was found to be 0.04 and 0.02 mM, respectively in heart and skeletal muscle. Using free ADP and measuring P-enolpyruvate, ATP, and Pyr in the tissues the mass action ratio was 4,382 for the pyruvate kinase biochemical reaction in heart and 3,050 in rat gastrocnemius. These values were compared with the calculated K' of 13,230 and 18,880 for heart and muscle, respectively (Table IV).

DISCUSSION

Thermodynamics of the Pyruvate Kinase Reaction—Over the past 40 years knowledge of the thermodynamics of the pyruvate reaction has relied to a very large extent on the early studies of McQuate and Utter (7). The equilibrium is complex, because the reaction lies very far to the right making it tech-

nically difficult to measure low levels of P-enolpyruvate and ADP in the presence of high concentrations of pyruvate. Using methodologies and a system of equations described in earlier studies, we report an enthalpy ($\Delta H'^{\circ}$) for the biochemical reaction (Reaction 1) of -4.31 kJmol^{-1} over the temperature range of 5 to 45 °C. This enthalpy value is in good agreement with the recalculated value of -4.7 kJmol^{-1} (pH 8.03, 25 °C) in the calorimetric study of Cheer *et al.* (29) (see Goldberg and Tewari; 30). A low negative $\Delta H'^{\circ}$ shows that the pyruvate kinase reaction is predominately entropically driven in the ATP direction, with the enthalpy term contributing only 16% to the overall $\Delta G'^{\circ}$ (Table II). This is in contrast to the thermodynamics of the creatine kinase and arginine kinase biochemical reactions, which are predominately enthalpy-driven (19, 20, 31). The differences demonstrate that pyruvate kinase reaction has very different transphosphorylation mechanisms compared with creatine kinase or arginine kinase. The relationship between K' and K_{ref} can be used to calculate K' for the pyruvate kinase reaction at differing pH and free $[Mg^{2+}]$ over the phys-

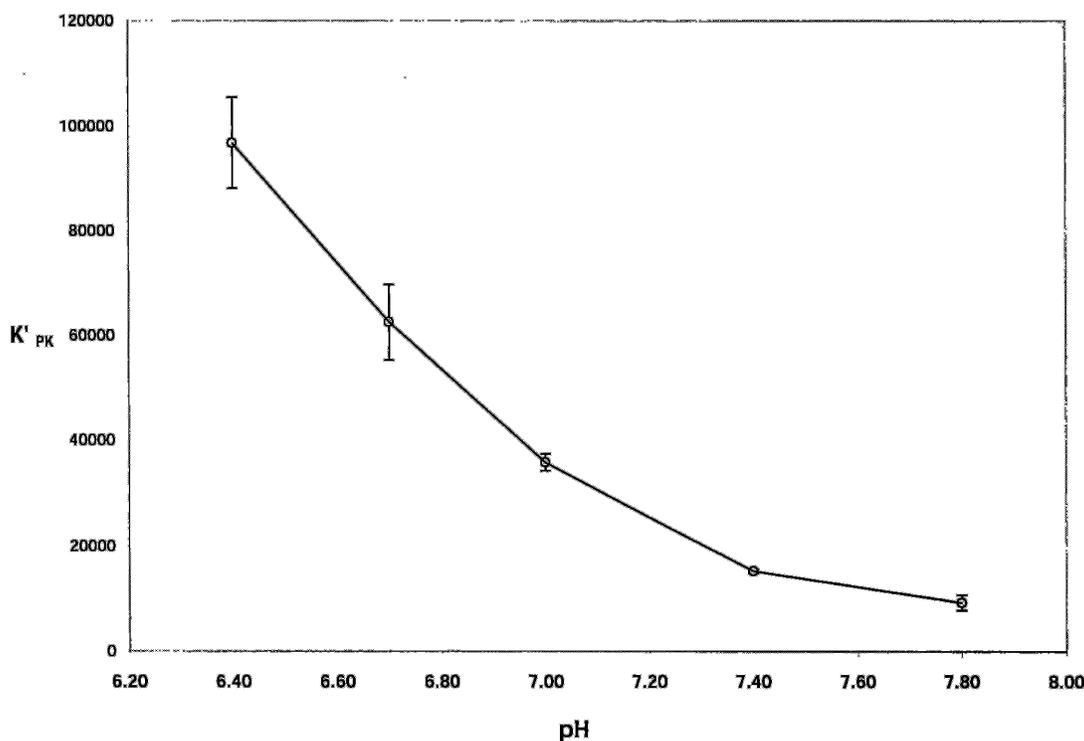


FIG. 2. Variation of K'_{PK} with pH. Each point represents the mean \pm S.E. ($n = 6$), $T = 25^\circ\text{C}$, free $[\text{Mg}^{2+}] = 1.0\text{ mM}$, and $I = 0.25\text{ M}$. (see "Materials and Methods" for details).

TABLE III

Calculation of free cytosolic [ADP] in rat heart and gastrocnemius skeletal muscle *in vivo*

All metabolites are expressed in mM intracellular water. Free cytosolic [ADP] was calculated using the creatine kinase equilibrium (see "Materials and Methods"). [Creatine] was calculated by [Total Creatine] - [Phosphocreatine]. Conversion of total tissue content ($\mu\text{mol/g}$ wet weight) into intracellular concentrations ($\mu\text{mol/ml}$) was performed using total tissue water (g/g wet weight) and intracellular space *in vivo* values of 0.79 and 0.581 (73%) for the rat heart and 0.76 and 0.64 (84%) for gastrocnemius muscle (22). Values are mean \pm S.E.

| Tissue | n | pH | Free $[\text{Mg}^{2+}]$ | K'_{CK} | ATP | PCr | Total Cr | Cr | Free [ADP] |
|-----------------|-----|------------------|-------------------------|------------|-----------------|-----------------|----------------|-----------------|------------------|
| Heart | 7 | 7.32 ± 0.04 | 0.46 ± 0.03 | 65 ± 8 | 8.28 ± 0.31 | 17.0 ± 0.28 | 22.3 ± 0.9 | 5.30 ± 1.07 | 0.04 ± 0.007 |
| Skeletal muscle | 7 | 7.18 ± 0.015 | 0.57 ± 0.02 | 95 ± 4 | 9.45 ± 0.26 | 36.5 ± 1.70 | 44.6 ± 1.0 | 8.10 ± 1.23 | 0.02 ± 0.005 |

TABLE IV

Assessment of the position of the pyruvate kinase equilibrium in rat heart and gastrocnemius skeletal muscle *in vivo*

Tissue mass action ratios are expressed in the ATP direction and compared with the apparent K'_{PK} adjusted to tissue pH and free $[\text{Mg}^{2+}]$ at 38°C and $I = 0.25\text{ M}$. All metabolites are expressed in $\text{mM} \pm$ S.E. Free [ADP] was calculated from the creatine kinase equilibrium (see Table III and "Materials and Methods"). Conversion of total tissue content ($\mu\text{mol/g}$ wet weight) into intracellular concentrations ($\mu\text{mol/ml}$) was performed using total tissue water (g/g wet weight) and intracellular space *in vivo* values of 0.79 and 0.581 (73%) for the rat heart and 0.76 and 0.64 (84%) for gastrocnemius muscle (27).

| Tissue | n | ADP | PEP | ATP | Pyr | Tissue mass action ratio [Pyr][ATP]/[PEP][ADP] | K'_{PK} adjusted to pH and free $[\text{Mg}^{2+}]$ of heart and muscle |
|-----------------|-----|------------------|-------------------|-----------------|------------------|---|--|
| | | <i>mM</i> | | | | | |
| Heart | 7 | 0.04 ± 0.008 | 0.010 ± 0.002 | 8.28 ± 0.82 | 0.11 ± 0.009 | $4,382 \pm 1550$ | 13,230 (3-fold) |
| Skeletal muscle | 6 | 0.02 ± 0.005 | 0.018 ± 0.002 | 9.45 ± 0.68 | 0.11 ± 0.009 | $3,050 \pm 533$ | 18,880 (6.2-fold) |

iological range at 38°C and $I = 0.25\text{ M}$. This relationship is shown below in Equation 3,

$$K'_{PK} = K_{ref}[\text{H}^+]$$

$$\frac{1 + \frac{[\text{H}^+]}{K_{aATP}} + (K_{bMgATP}[\text{Mg}^{2+}] + \frac{(K_{bMgHATP}[\text{H}^+][\text{Mg}^{2+}])}{K_{aATP}})}{\left(1 + \frac{[\text{H}^+]}{K_{aADP}} + (K_{bMgADP}[\text{Mg}^{2+}] + \frac{(K_{bMgHADP}[\text{H}^+][\text{Mg}^{2+}])}{K_{aADP}})\right) \left(1 + \frac{[\text{H}^+]}{K_{aPEP}} + (K_{bMgPEP}[\text{Mg}^{2+}])\right)}$$

(Eq. 3)

where K_{ref} is 9.53×10^{10} at 38°C , and the acid-dissociation and magnesium binding constants are adjusted to 38°C (23). The K_d for P-enolpyruvate ($\text{HP-enolpyruvate}^{2-} = \text{P-enolpyruvate}^{3-}$

+ H^+) was 4.2×10^{-7} at 38°C and $I = 0.25$, and the magnesium binding constant for P-enolpyruvate ($K_{bP-enolpyruvate}^{-1}$) ($\text{Mg}^{2+} + \text{P-enolpyruvate}^{3-} = \text{MgP-enolpyruvate}^{-1}$) was 2.2×10^2 at 38°C and $I = 0.25\text{ M}$. Values for K' at different pH and free $[\text{Mg}^{2+}]$ over the physiological range is presented in Table V.

Physiological Significance of the Thermodynamic Data and Reversal of Glycolysis—Most biochemical texts refer to the pyruvate kinase reaction as a highly exergonic reaction positioned far from equilibrium (11, 16, 32). Newsholme and Start (33) in *Regulation in Metabolism* state, "The mass action ratio for pyruvate kinase indicate that this enzyme catalyzes a re-

TABLE V
Effect of pH and free Mg^{2+} concentrations on K_{PK} at 38 °C and $I = 0.25$ M. $K_{PK} = [Pyr][ATP]/[PEP][ADP]$

| pH | Free $[Mg^{2+}]$ (mM) | | | | | | | | | | | | | |
|-----|-----------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 |
| 6.4 | 22951 | 31021 | 38033 | 44153 | 49517 | 54234 | 58395 | 62073 | 65331 | 68223 | 70792 | 73076 | 75108 | 76916 |
| 6.5 | 19890 | 27731 | 34450 | 40238 | 45247 | 49600 | 53395 | 56712 | 59617 | 62166 | 64405 | 66373 | 68103 | 69623 |
| 6.6 | 17021 | 24451 | 30732 | 36076 | 40647 | 44573 | 47958 | 50885 | 53421 | 55622 | 57534 | 59195 | 60637 | 61889 |
| 6.7 | 14398 | 21272 | 27010 | 31835 | 35917 | 39387 | 42347 | 44881 | 47055 | 48921 | 50525 | 51903 | 53086 | 54099 |
| 6.8 | 12054 | 18272 | 23404 | 27674 | 31251 | 34262 | 36807 | 38965 | 40799 | 42359 | 43686 | 44813 | 45770 | 46579 |
| 6.9 | 10000 | 15513 | 20016 | 23728 | 26810 | 29382 | 31538 | 33352 | 34879 | 36167 | 37252 | 38165 | 38931 | 39571 |
| 7.0 | 8231 | 13031 | 16918 | 20095 | 22712 | 24881 | 26685 | 28191 | 29450 | 30503 | 31383 | 32117 | 32726 | 33229 |
| 7.1 | 6730 | 10845 | 14151 | 16835 | 19030 | 20838 | 22332 | 23572 | 24601 | 25456 | 26165 | 26751 | 27234 | 27628 |
| 7.2 | 5473 | 8953 | 11730 | 13972 | 15795 | 17288 | 18515 | 19528 | 20364 | 21054 | 21623 | 22090 | 22471 | 22778 |
| 7.3 | 4430 | 7340 | 9649 | 11504 | 13005 | 14228 | 15230 | 16052 | 16728 | 17283 | 17738 | 18109 | 18409 | 18649 |
| 7.4 | 3573 | 5982 | 7886 | 9409 | 10636 | 11633 | 12446 | 13110 | 13654 | 14099 | 14462 | 14756 | 14993 | 15181 |

action which is removed from equilibrium.” Albert Lehninger (34) in *Principles of Biochemistry* wrote, “The (PK) reaction also tends to go far to the right under standard conditions because the ΔG° P-enolpyruvate is much larger than ΔG° ATP hydrolysis. The reaction is irreversible in the cell.” More recently Voet *et al.* (35) in *Fundamentals of Biochemistry* wrote, “Only three reactions, those catalyzed by hexokinase, phosphofructokinase and pyruvate kinase, . . . are nonequilibrium reactions of glycolysis and candidates for flux control points.”

Our study shows that the pyruvate kinase reaction is poised much closer to near-equilibrium than believed previously. On the basis of NMR and metabolic measurements, the mass action ratio in heart and resting skeletal muscle is only 3- to 6-fold from the equilibrium constant of the reaction determined on the bench and adjusted to the same pH, free $[Mg^{2+}]$, and temperature of the tissues (see Tables IV and V). The mass action ratio calculation assumes that all the measured or calculated reactants *in situ* are thermodynamically free with no binding, or compartmentation occurs in the tissue. The striking aspect of the comparison is the close agreement reached between the observed and theoretical constants in heart and skeletal muscle. The thermodynamic data indicate that a fall in intracellular pH (and a rise in free $[Mg^{2+}]$), such as during high intensity exercise, hypoxia, or ischemic injury, would lead to the equilibrium shifting further to the right. If the pH fell by 0.6 units (7.3 to 6.7), and free $[Mg^{2+}]$ rose by 0.2 mM (0.5 to 0.7 mM), the equilibrium position would increase over 3-fold from around 14,000 to 45,000 (Table V). Conversely during recovery, as tissue pH and free $[Mg^{2+}]$ returned to “resting” values, the equilibrium position would be poised much closer to near-equilibrium. Thus, on the basis of our thermodynamic analysis, we propose that the equilibrium position during anaerobic exercise would favor strongly ATP and pyruvate formation, and during recovery it would be more favorable to permit replenishment of glycogen stores.

However, the thermodynamics (*i.e.* the extent and direction of a reaction) is not the only consideration required to assess the role of pyruvate kinase in the reversal of glycolysis in intact tissues. Another important factor is kinetic constraints over the activity of the enzyme. If the activity of pyruvate kinase is not sufficiently high, reversal may be limited kinetically, even if the equilibrium is favorable. In support of the earlier work of Lardy and Ziegler (4), Krimsky (6), and McQuate and Utter (7), Dyson *et al.* (8) have shown that the reversal of pyruvate kinase and glycolysis is kinetically feasible in muscle. Using a coupled reaction that removed P-enolpyruvate, they showed that the enzyme could catalyze the phosphorylation of pyruvate at a maximum velocity of about $6 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (6, 8). Dyson *et al.* (8) further noted that given the high activity in skeletal muscle, the physiological significance of the reverse reaction of pyruvate kinase in muscle has been underestimated, particularly its role in glycogen synthesis (8). Newsholme and Start

(33) also noted the extremely high maximal activity of pyruvate kinase relative to the rate-controlling phosphofructokinase in muscle, heart, and brain extracts (up to 10-fold higher), which suggests that the enzyme must be strongly inhibited, particularly in muscle. The maximal activity of pyruvate kinase in rat and rabbit gastrocnemius is about 800 and 1100 μmol of substrate $\text{g}^{-1} \text{wet wt min}^{-1}$, respectively (36). Because there is around 2 mg of enzyme per g wet weight muscle, the reverse rate of Dyson *et al.* (8) equates to $12 \mu\text{mol min}^{-1} \text{g}^{-1} \text{wet weight muscle}$, which is around 1 to 2% of the maximal velocity of the enzyme.

Having argued that the thermodynamics and kinetics of pyruvate kinase are favorable under some conditions, an important question remains: “How does the rate of pyruvate kinase reversal compare with the observed rate of glycogen synthesis in skeletal muscle following exercise?” The highest glycogen synthesis rate from lactate following short term high intensity exercise was reported by Hermansen and Vaage (13) in human quadriceps. They reported a value of $0.56 \mu\text{mol glucosyl units g}^{-1} \text{wet wt min}^{-1} \text{muscle}$, which translates to about $17 \mu\text{mol g}^{-1}$ during 30 min of recovery after three bouts of maximal cycling (13). In contrast, following prolonged aerobic exercise, this rate of glycogen resynthesis is over an order of magnitude lower with rates ranging from 0.025 to $0.03 \mu\text{mol glucosyl units g}^{-1} \text{wet wt min}^{-1} \text{muscle}$ (37). Even at the highest reported glycogen resynthesis rates, the estimate of Dyson *et al.* (8) of $12 \mu\text{mol min}^{-1} \text{g}^{-1} \text{wet weight muscle}$ for pyruvate phosphorylation is ample to explain the observed rates of glycogen synthesis *in vivo*.

McLane and Hollozy (14) also argued that pyruvate kinase reversal could account for glycogen formation from lactate in three types of rat skeletal muscle, as has Hochachka and colleagues (38) in fish white muscle, which, like other muscles, lacks the bypass enzymes pyruvate carboxylase and P-enolpyruvate carboxykinase. More recently, Donovan and Pagliasotti (18) also have concluded that PEPCK is not involved in mammalian skeletal muscle glyconeogenesis and concluded pyruvate must be reversed to explain the rates of glycogen replenishment. They suggested that P-enolpyruvate formation in skeletal muscle glyconeogenesis occurs by reversal of the pyruvate kinase reaction (18). On the basis of the available literature, and because malic enzyme appears to thermodynamically favor the decarboxylation of malate to pyruvate (39), it is now generally believed that malic enzyme, malate dehydrogenase, and P-enolpyruvate carboxykinase are specific to glucogenic tissues such as liver and kidney and not skeletal muscle or heart. However, the thermodynamics of the pyruvate kinase reaction reported in this study apply equally to all tissues. What remains to be established is the mass action ratios in liver and kidney.

On the question of the role of pyruvate kinase and the reversal of glycolysis *in situ*, it would appear that intracellular pH

and free $[Mg^{2+}]$ must return to, or at least approach, pre-exercise or pre-ischemic values, before the equilibrium favors reversal. Otherwise at low pH or high free $[Mg^{2+}]$, the equilibrium position will work against pyruvate phosphorylation and therefore glycogen synthesis. Future studies are required to test this hypothesis to achieve the full physiological significance of the thermodynamic and kinetic properties of pyruvate kinase. Of the twelve reactions that comprise the glycogenolytic pathway, nine have traditionally been considered near-equilibrium, and three are displaced far from equilibrium. The three non-equilibrium reactions are glycogen phosphorylase (EC 2.4.1.1), phosphofructokinase-I (EC 2.7.1.11), and pyruvate kinase. Our thermodynamic data show that the pyruvate kinase reaction is much closer to equilibrium in resting skeletal muscle and heart than otherwise believed and implies regulatory control of glycogenolysis at the glycogen phosphorylase and phosphofructokinase steps and at hexokinase and phosphofructokinase steps in the case of glycolysis. Finally, around 30 years ago Newsholme and Start (33) recognized some of the paradoxical aspects of pyruvate kinase when they wrote, "Another problem enzyme is pyruvate kinase; the mass action ratio strongly indicates that it catalyzes a non-equilibrium reaction whereas its maximal catalytic activity suggests otherwise." Our study indicates that, under some circumstances, the pyruvate kinase reaction can approach near-equilibrium and that the high maximal activity is significant to the reverse reaction and glycogen replenishment.

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