Bovine Pyruvate Kinases

I. PURIFICATION AND CHARACTERIZATION OF THE SKELETAL MUSCLE ISOZYME*

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SUMMARY

Pyruvate kinase has been purified from bovine skeletal muscle by a procedure that includes only heat, ammonium sulfate fractionation, and chromatography on carboxymethyl Sephadex. Crystallization was accomplished by dialysis at 4° against 47% saturated ammonium sulfate. Since this simple purification scheme, with only slight modifications, has also been used to isolate turkey and rabbit muscle pyruvate kinase, it may be generally applicable to the isolation of muscle pyruvate kinase from birds and mammals.

Bovine muscle pyruvate kinase prepared by the procedure described here was found to be homogeneous, as determined by disc gel electrophoresis at pH 9.5, gel electrophoresis in the presence of sodium dodecyl sulfate, sedimentation velocity and sedimentation equilibrium, isoelectric focusing, and immunodiffusion. It sediments at 9.9 S (s20,w) and has a maximum velocity of 400 μmoles per min per mg or more at 25°, or 2.4 times that value at 37°. The enzyme has maximal activity at pH 7.1, with an apparent Michaelis constant for phosphoenolpyruvate of 0.04 mM or less, and for ADP of 0.4 mM. Its isoelectric pH during electrofocusing is 8.9.

Sedimentation equilibrium yielded a molecular weight of 230,000 with a range of ±3,000 in dilute phosphate buffer, and 57,000 ± 1,500 in 3.5 M guanidine hydrochloride, using a partial specific volume of 0.740 ml per g calculated from the amino acid composition. The molecular weight in guanidine hydrochloride, confirmed by gel electrophoresis in sodium dodecyl sulfate, indicates the presence of four polypeptide chains in the native enzyme. Binding studies suggest a total of four phosphoenolpyruvate binding sites, or one per cubulin. Ion sensitivities and other chemical and physical parameters are very similar to those reported for rabbit muscle pyruvate kinase.

The glycolytic enzyme, pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), has been found in all cell types studied. At least three electrophoretically distinct mammalian isozymes have been identified in adult rat tissues (1-10). Imamura and Tanaka (10) have designated these isozymes as M1, M2, and L, while Susor and Rutter (4, 9) use A, C, and B, respectively, for the same isozymes.

Type M1 pyruvate kinase is apparently found alone in skeletal muscle. It is also the major isozyme of heart and brain. Type L is the major pyruvate kinase isozyme of liver and a minor component of the kidney. Type M2 has been found in all mammalian tissues examined except skeletal muscle and erythrocytes. As the erythrocyte enzyme appears to be electrophoretically different from the preceding isozymes, Susor and Rutter designate it as type D.

Mammalian skeletal muscle pyruvate kinase (type M1) obeys standard Michaelis-Menten kinetics under most conditions and is unaffected by fructose 1,6-diphosphate. On the other hand, the liver isozyme (type L) has a sigmoidal velocity profile with respect to phosphoenolpyruvate and is activated by fructose 1,6-diphosphate. These divergent kinetic properties suggest that hybrids of M1 and L should be useful in helping to define the structure and different physiological functions of these isozymes. In addition, because the hybrids would have subunits derived from an allosteric and a non-allosteric enzyme, they should yield valuable information concerning the mechanism of allostery as it applies to the type L isozyme.

Since considerable quantity of enzyme would be desirable for a thorough study of the hybrids, there is a definite advantage in obtaining tissue from a large animal. In addition, there are obvious conveniences involved in using animals that are slaughtered commercially. Accordingly, we have proceeded to isolate the skeletal muscle and liver pyruvate kinase isozymes from beef, preparatory to a study of the hybrids.

This paper describes a simple procedure that we have found to be useful for the purification of bovine skeletal muscle (type M1) pyruvate kinase and, with slight modifications, for the purification of muscle pyruvate kinase from chicken, turkey, and rabbit. We also report the results of various kinetic, physical, and chemical studies on bovine skeletal muscle pyruvate kinase, and briefly compare this enzyme with the extensively studied skeletal muscle pyruvate kinase from rabbits.

MATERIALS AND METHODS

The enzyme was isolated from bovine neck muscle, obtained from a slaughterhouse shortly after the animal was killed. Ammonium sulfate was enzyme grade from Mann; Bio-Gel was...
obtained from Bio-Rad Laboratories; substrates and lactate dehydrogenase were obtained from Sigma Chemical Co.; guanidine hydrochloride was spectroscopic grade, obtained from Heico Inc., Delaware Water Gap, Pa. All other chemicals, except where otherwise noted, were standard reagent quality. Distilled, deionized water was used for all solutions.

Assay

Pyruvate kinase activity was generally assayed by coupling the pyruvate kinase reaction to that of lactate dehydrogenase according to the method of Bücher and Pfleiderer (11). Cuvettes of 4-mm sample width and 10-mm light path were used. The standard assay was carried out at pH 7.0 in 1 ml of 0.05 M imidazole-HCl, 0.10 M KCl, 10 mM MgCl₂, 1 mM phosphoenolpyruvate, 2 mM ADP, 0.16 mM NADH, and approximately 20 units (micromoles per min) of lactate dehydrogenase. A Beckman Acta III recording spectrophotometer was used to measure the decrease in absorbance at 340 nm. Temperature was controlled at 25° by means of a circulating water bath.

Protein concentrations in the less pure fractions were determined by the method of Folin-Ciocalteau, as described by Clark (12), using bovine serum albumin as a standard. In the more highly purified samples, protein concentrations were estimated from the absorbance at 280 nm, using an extinction coefficient (E₄₈₅) of 0.55, determined in this study.

Enzyme Purification

All steps were carried out at 0°-4° unless otherwise stated. Certain of the steps were adapted from procedures used by others (13, 14) in isolating the rabbit muscle enzyme. The procedure is summarized in Table I.

Step 1: Extraction—After removing fat, the muscle was passed through a precooked meat grinder, then blended at high speed in an Osterizer food blender for 30 s with 2 ml of 1% EDTA per g of muscle. The mixture was stirred for 1 hour before centrifuging at 8000 × g for 20 min. The supernatant was filtered through a loose plug of glass wool and saved; the precipitate was discarded.

Step 2: Heat Treatment—The filtered supernatant from Step 1 was brought to 0.02 M imidazole by the addition of appropriate quantities of 1.0 M imidazole-HCl buffer, pH 7.0. The final pH was adjusted to 7.0 with HCl or KOH if necessary. The solution was heated rapidly to 60° by swirling in an 80° water bath, maintained at 60° for 5 min with constant agitation, and then rapidly cooled to at least 10° by swirling in an ice bath. The resulting suspension was centrifuged for 20 min at 10,000 × g, 0°. The precipitate was discarded.

Step 3: Ammonium Sulfate Fractionation—The supernatant from the heat step was brought to 54% ammonium sulfate saturation at 0° (i.e. in an ice water bath) by the gradual addition, with constant stirring, of 320 g of solid ammonium sulfate per liter of extract. Stirring was continued for 30 min following ammonium sulfate addition, after which the mixture was centrifuged at 10,000 × g for 20 min. The precipitate was discarded and the supernatant brought to 67.5% ammonium sulfate saturation by the addition of 93.5 g of solid ammonium sulfate per liter of 54% saturated solution, following the same procedure for the addition of ammonium sulfate as indicated above. The solution was centrifuged at 10,000 × g for 20 min, after which the supernatant was discarded. The precipitate was resuspended at a concentration of 20 to 40 mg per ml in 80% saturated ammonium sulfate containing 0.01 M β-mercaptoethanol. Pyruvate kinase at this stage of purification is stable for several days, at least, when stored as an ammonium sulfate suspension at 0°-4°.

Step 4: Carboxymethyl Sephadex Chromatography—Carboxymethyl Sephadex, type C-50 (Pharmacia Fine Chemicals) was prepared for chromatography in the following manner. The gel was allowed to swell 48 hours in deionized water, washed successively in a Buchner funnel with several volumes of 1 M KCl, then 0.1 M KOH, and finally 0.1 M HCl, rinsing with deionized water between each step. The gel was then equilibrated with 0.03 M potassium phosphate buffer, 0.01 M β-mercaptoethanol, pH 6.0, before packing the column (2.5 × 40 cm). A peristaltic pump was used to keep the flow rate constant at about 20 ml per hour during chromatography. Up to 500 mg of protein from the 67.5% ammonium sulfate precipitate, dialyzed overnight versus the equilibrating buffer, were chromatographed at one time. The protein was washed into the column with approximately 75 ml of equilibrating buffer before applying a linear gradient of 0 to 0.3 M KCl in the same buffer. The total volume of the gradient was 1 liter. A typical elution pattern is shown in Fig. 1. Fractions having the highest specific activities, generally greater than 150 μmoles per min per mg at 25°, were pooled and dialyzed versus 80% ammonium sulfate, 0.01 M β-mercaptoethanol, pH 7.0, and stored at 0°-4°. The enzyme is stable in this condition for several months, at least.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total pyruvate kinase activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>μmoles/min</td>
<td>mg</td>
<td>μmoles/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>1. Extract from 232 g of neck muscle</td>
<td>450</td>
<td>18,000</td>
<td>5360</td>
<td>3.4</td>
<td>100</td>
</tr>
<tr>
<td>2. Heat step</td>
<td>440</td>
<td>17,200</td>
<td>2680</td>
<td>6.4</td>
<td>95</td>
</tr>
<tr>
<td>3. Ammonium sulfate</td>
<td>27</td>
<td>14,500</td>
<td>383</td>
<td>37.7</td>
<td>80</td>
</tr>
<tr>
<td>4. Cm-Sephadex chromatography</td>
<td></td>
<td>6,090</td>
<td>26.1</td>
<td>233</td>
<td>34</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatography of bovine skeletal muscle pyruvate kinase (PK) on carboxymethyl Sephadex, C-50. The column, which was prepared and eluted as described under "Materials and Methods," was loaded with 262 mg of protein, with a specific activity of 36 units per mg. (One unit converts 1 μmole of substrate per min.) The fractions are 4.4 ml each.
Bovine muscle pyruvate kinase was crystallized at concentrations of 20 to 10 mg per ml by the very slow addition of saturated ammonium sulfate (pH 7.0) to incipient turbidity, or by placing the protein solution in a dialysis bag and letting ammonium sulfate at 47% of saturation diffuse into the bag without stirring. The crystals had the appearance of small needles.

In uncrystallized preparations, trace amounts of adenylate kinase or lactate dehydrogenase were sometimes detected after crystallization. Gel filtration on Sephadex G-200, applying 10 to 20 mg of protein to a column (2.5 × 50 cm), and eluting it at 10 to 15 ml per hour with 0.05 M potassium phosphate buffer, 0.01 M β-mercaptoethanol, pH 7.0

Amino Acid Analysis

Amino acid analyses were performed on a Beckman 120B amino acid analyzer. The samples were hydrolyzed at 110° in 6 N HCl in sealed, evacuated vials for 20, 42, or 67 hours before being analyzed. The values for threonine and serine were extrapolated to zero time, assuming a first order decay. The highest values obtained in the three analyses were taken for valine and isoleucine. Cystine was determined as cysteic acid, using a performic acid oxidation prior to hydrolysis. Tryptophan was measured by the method of Edelhoch (15). Other numbers in the "corrected" column of Table II were averages of the three hydrolysis periods.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Bovine 2 hr</th>
<th>Bovine 42 hr</th>
<th>Bovine 67 hr</th>
<th>Corrected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rabbit&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>147.0</td>
<td>149.8</td>
<td>153.4</td>
<td>150</td>
<td>148</td>
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<tr>
<td>Histidine</td>
<td>68.4</td>
<td>59.2</td>
<td>61.5</td>
<td>60</td>
<td>62</td>
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<tr>
<td>Ammonia</td>
<td>225.1</td>
<td>213.2</td>
<td>246.1</td>
<td>228</td>
<td>107</td>
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<tr>
<td>Arginine</td>
<td>146.7</td>
<td>126.6</td>
<td>125.1</td>
<td>133</td>
<td>113</td>
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<tr>
<td>Cysteic acid</td>
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<td>111.2</td>
<td>108.9</td>
<td>106.9</td>
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<tr>
<td>Glutamic acid + asparagine</td>
<td>194</td>
<td>194</td>
<td>194</td>
<td>194</td>
<td>202</td>
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<tr>
<td>Threonine</td>
<td>101.4</td>
<td>93.1</td>
<td>83.7</td>
<td>109</td>
<td>104</td>
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<tr>
<td>Serine</td>
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<td>88.9</td>
<td>72.5</td>
<td>126</td>
<td>120</td>
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<tr>
<td>Pheldecostic acid + glutamine</td>
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<td>130.0</td>
<td>127.0</td>
<td>127</td>
<td>127</td>
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<tr>
<td>Proline</td>
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<td>89.6</td>
<td>87.5</td>
<td>89</td>
<td>92</td>
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<tr>
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<td>161.7</td>
<td>165.3</td>
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<tr>
<td>Alanine</td>
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<td>218.2</td>
<td>221.6</td>
<td>219</td>
<td>244</td>
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<tr>
<td>Valine</td>
<td>187.4</td>
<td>174.2</td>
<td>170.3</td>
<td>174</td>
<td>185</td>
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<tr>
<td>Methionine</td>
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<td>60.5</td>
<td>70.2</td>
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<tr>
<td>Isoleucine</td>
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<td>126.0</td>
<td>133.6</td>
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<tr>
<td>Leucine</td>
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<td>157.0</td>
<td>164.0</td>
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<tr>
<td>Tyrosine</td>
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<td>35.9</td>
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<tr>
<td>Phenylalanine</td>
<td>84.2</td>
<td>66.7</td>
<td>66.7</td>
<td>65</td>
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<tr>
<td>Tryptophan</td>
<td>86.6</td>
<td>86.6</td>
<td>86.6</td>
<td>86</td>
<td>86</td>
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</tbody>
</table>

<sup>a</sup> See "Materials and Methods" for explanation. Values are moles of residue per 230,000 g of protein, rounded to the nearest residue.

<sup>b</sup> Values are moles of residue per 237,000 g of protein, as reported by Cottam et al. (30).

Ultracentrifuge Studies

A model E analytical ultracentrifuge (Spinco Division, Beckman Instruments) was employed, using electrophoretic optics for sedimentation velocity and interference optics for sedimentation equilibrium. The interference system was aligned and focused according to the procedure of Richards et al. (16, 17). Alignment was checked at frequent intervals with the test described by Dyson (18). Photographic plates were read on a Nikon 6C comparator equipped with micrometer digitizers (L & W Electronics, Houston, Tex.) communicating directly with the time-shared Control Data Corp. 3300 computer on which subsequent calculations were performed. Most sedimentation equilibrium experiments followed the meniscus depletion procedure of Yphantis (19), sometimes modified in a way similar to that suggested by LaBar (20). The protein solution was dialyzed overnight at 4° when dilute buffers were employed for sedimentation equilibrium, and for 2 days at 25° prior to sedimentation equilibrium in guanidine hydrochloride. The partial specific volume of the enzyme was estimated from its amino acid content (21).

Extinction Coefficient

A solution containing several milligrams of pyruvate kinase was exhaustively dialyzed against 0.01 M ammonium acetate, pH 7.0. The absorbance of the dialyzed solution was read at 280 nm and a synthetic boundary experiment was performed in the analytical ultracentrifuge to establish the number of fringes per concentration unit. A carefully measured quantity was dried at 85° in a tared vial, which was then placed in a drying pistol containing P2O5. The pistol was evacuated and brought to 100° with a steam bath. At intervals of 1 or 2 days, the apparatus was allowed to cool to ambient temperature, air was admitted through an anhydrous CaCl2 drying tube, and the vial was quickly removed and weighed on a microbalance. (The air in the balance case was dried with silica gel.) The experiment was continued until successive weighing produced the same value. A parallel experiment using dialysate only was carried out to correct for nonvolatile components in the ammonium acetate, the quantity of which was found to be negligible.

Electrophoresis and Isoelectric Focusing

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the procedure of Shapiro et al. (22), as modified by Weber and Osborn (23), using Eastman reagents. Standard disc gel electrophoresis at pH 9.5 followed the procedure summarized by Gabriel (24). Isoelectric focusing was carried out with an apparatus and reagents sold by LKB Instruments, Inc.

Binding Studies

Substrate binding studies were carried out according to the method of Castellino and Barker (25), using a column (0.9 × 120 cm) treated with a 1% solution of dimethylglucamine in benzene, then dried and washed with a detergent. Bio-Gel P-6 or P-10, 50 to 100 mesh, was washed first with 25% methanol, then with distilled deionized water, then in 0.5 M KCl, and finally with several volumes of imidazole-KCl buffer (0.05 M imidazole-Cl, 0.1 M KCl, 0.01 M MgCl2, pH 7.0) before equilibrating the column with the desired concentration of phosphoenolpyruvate dissolved in the imidazole-KCl buffer. Binding studies were performed at 4° in order to reduce a slow hydrolysis of phosphoenolpyruvate to pyruvate seen at room temperature.
In some experiments, MgCl₂ was replaced by EDTA for the same reason.

Approximately 20 mg of pyruvate kinase in a volume of 1 to 2 ml were dialyzed overnight against the imidazole-KCl buffer. Phosphoenolpyruvate was added to the enzyme solution immediately before applying it to the column. Columns were eluted with imidazole-KCl buffer of the same phosphoenolpyruvate concentration, maintaining a rate of approximately 0.3 ml per min and collecting fractions of 0.5 to 1.0 ml. Enzyme concentrations were determined by catalytic activity and by absorbance at 280 nm; phosphoenolpyruvate concentrations were determined with a coupled assay containing 0.05 M imidazole-HCl, 0.1 M KCl, 0.01 M MgCl₂, 2 mM ADP, 0.14 mM NADH, pH 7.0, plus 0.1 unit (micromoles per min) or more of lactate dehydrogenase and pyruvate kinase, using the decrease in absorbance at 340 nm after correcting for volume changes.

Substrate binding studies were also carried out with an analytical ultracentrifuge, using a method similar to that of Hirsch-Kolb et al. (26), but without radioactive labels. Pyruvate kinase solutions of 5 to 10 mg per ml were dialyzed overnight against the imidazole-KCl buffer described above. Just before loading the partitioned centrifuge cell (both fixed partition and movable partition centerpieces were used), phosphoenolpyruvate was added to the desired level. The sample was centrifuged at 60,000 rpm at 4°C in a Beckman model E analytical centrifuge for 75 min, allowing the protein peak to move into the lower third of the cell. The centrifuge was stopped without braking. Samples withdrawn from both the upper and lower sections were analyzed for pyruvate kinase and phosphoenolpyruvate. Corrections for the slight sedimentation of phosphoenolpyruvate were made by performing control experiments in which pyruvate kinase was omitted.

**Immunological Studies**

Immunizations and agar diffusion tests were graciously performed by Dr. M. Rittenberg of the University of Oregon Medical School. Rabbits were immunized against bovine muscle pyruvate kinase by injecting them (either in the footpad or subcutaneously) with 0.65 mg of the enzyme in 0.85% NaCl solution, mixed with 0.65 mg of methylated serum albumin (27) in Freund's complete adjuvant. Two injections were made at least a month apart. The rabbits were bled from an ear vein 1 week after the second immunization. In a quantitative precipitin test, the immune serum completely removed pyruvate kinase activity from the supernatant. Agar diffusion tests were carried out on microscope slides by the method of Hartmann and Toelliez (28).

**RESULTS**

**Homogeneity**—Homogeneity of bovine skeletal muscle pyruvate kinase, prepared by the procedure described here, was established by the following criteria. (a) A single, symmetrical peak was observed during sedimentation in the analytical ultracentrifuge (Fig. 2). (b) The dependence of sedimentation coefficient on concentration is consistent with a relatively compact, homogeneous, nonassociating protein (Fig. 3). (c) Sedimentation equilibrium of the native enzyme yielded straight lines when the logarithm of concentration was plotted against the square of radial position (Fig. 4). (d) A similar linearity was found after reaching sedimentation equilibrium in 3.5 M guanidine hydrochloride (with 0.01 M β-mercaptoethanol), a medium that dissociates this and most other multisubunit proteins (Fig. 5). (e) A single band was found after disc gel electrophoresis. (f) A single band was found after gel electrophoresis in the presence of sodium dodecyl sulfate. (g) Isoelectric focusing in a sucrose gradient produced a single peak (Fig. 6). (h) A single precipitin band was found in agar diffusion tests, using antisemur from rabbits immunized with bovine pyruvate kinase prepared as above, and testing it against solutions of the same. (We note also that this antisemur, which removes bovine muscle pyruvate kinase from solution, fails to do the same for the rabbit enzyme.)

**Physical Properties**—The sedimentation coefficient extrapolated to infinite dilution (s²₀,w) was 9.94 S (Fig. 3), assuming a concentration dependence described by s²ₑ = s²₀,w (1 - kc). The value for k in this equation was 8 × 10⁻⁴ (milligrams per ml)⁻¹. The standard error in s²₀,w and k was 0.03 and 8 × 10⁻⁴, respectively, with a root mean square deviation in the data of 0.03 S.

The molecular weight from sedimentation equilibrium is 230,000 with an observed range of about ±5,000. These values are based on a partial specific volume of 0.70 ml per g, calcu-
The molecular weight of native bovine muscle pyruvate kinase was determined by ultracentrifugation and sedimentation equilibrium. The molecular weight, partial specific volume, and sedimentation coefficient yield a molecular frictional coefficient of $1.0 \times 10^{-7}$ g per s, a value that would be expected from an anhydrous sphere of radius 53 Å. The ratio of the two frictional coefficients, 1.3, could be accounted for by an anhydrous prolate ellipsoid with an axial ratio of 6.6, or by a spherical molecule containing 0.9 g of water per g of protein, or by a combination of hydration and asymmetry falling in between those two extremes (29).

Comparison of the molecular weight of the native enzyme with that of guanidine hydrochloride-dissociated material indicates that bovine skeletal muscle pyruvate kinase consists of four subunits, each with a molecular weight of 57,000. The range in the latter value, observed in various sedimentation equilibrium experiments performed in guanidine hydrochloride (using $0.740$ ml per g for the partial specific volume), was $\pm 1,000$. Essentially the same value for the subunit molecular weight was obtained from gel electrophoresis in the presence of sodium dodecyl sulfate.

**Chemical Properties**—The amino acid composition of the enzyme is presented in Table II. The isoelectric pH, as determined by isoelectric focusing, is 8.9 (see Fig. 6). From the dry weight determination, we find that a 1 mg per ml solution has an $A_{280}$ of 0.584 per cm and produces 3.88 fringes in a 12-mm synthetic boundary ultracentrifuge cell, using 0.01 M ammonium acetate at 20°, pH 7. The ratio $A_{280}/A_{260}$ is 1.71.

The results of binding studies carried out with gel columns under various conditions are presented in Table III. When phosphoenolpyruvate concentrations are high, up to 4 phosphoenolpyruvate molecules are bound per molecule of enzyme, or an average of one binding site per subunit. Similar results were obtained with binding studies carried out in the analytical ultracentrifuge. The data, however, are not extensive enough at present to warrant calculation of a binding constant.

Lineweaver-Burk plots for bovine pyruvate kinase are shown in Fig. 7. At 1.5 mM ADP, the apparent Michaelis constant for phosphoenolpyruvate was 0.04 mM, with some increase in higher values of ADP. At 0.5 mM phosphoenolpyruvate, the apparent $K_m$ for ADP was about 0.4 mM. Activities at 37° were found to be 2.4 times as great as the corresponding values at 25°. The pH optimum is 7.1 using the assay described under “Materials and Methods.” The activity falls to about half at $\pm 1$ pH unit from the optimum, using the same imidazole-KCl buffer.

Studies on the effect of monovalent and divalent cations indicate that both Mg$^{2+}$ and K$^+$ are required for maximal activity, although the addition of 0.02 M NH$_4^+$ to the assay medium was approximately 62% as effective as 0.1 M K$^+$ in activating the enzyme (Table IV). A 9% activation was obtained with Na$^+$ as the monovalent cation, although we have not eliminated the possibility that this activation was due to a small amount of K$^+$ contamination in the NaCl. No pyruvate kinase activity was observed when either Mg$^{2+}$ or monovalent cations were omitted from the assay medium. Fructose 1,6-diphosphate, an allosteric activator of mammalian pyruvate kinase, was not an activator of the bovine enzyme.

**Table III**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[PEP]$^2$</th>
<th>[PEP]/[Enzyme]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-10 at 4°, 0.05 mM EDTA</td>
<td>0.61</td>
<td>3.6</td>
</tr>
<tr>
<td>P-10 at 4°, 0.10 mM EDTA</td>
<td>0.28</td>
<td>2.3</td>
</tr>
<tr>
<td>P-10 at 4°, 4.0 mM Mg$^{2+}$</td>
<td>0.50</td>
<td>3.8</td>
</tr>
<tr>
<td>P-10 at 4°, 4.0 mM Mg$^{2+}$</td>
<td>0.55</td>
<td>2.8</td>
</tr>
<tr>
<td>P-10 at 23°, 4.0 mM Mg$^{2+}$</td>
<td>0.51</td>
<td>3.6</td>
</tr>
<tr>
<td>P-5 at 23°, 4.0 mM Mg$^{2+}$</td>
<td>0.50</td>
<td>2.8</td>
</tr>
<tr>
<td>P-5 at 23°, 4.0 mM Mg$^{2+}$</td>
<td>0.46</td>
<td>3.2</td>
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* The brackets indicate concentration.
TABLE V
Comparison of bovine and rabbit skeletal muscle pyruvate kinases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bovine pyruvate kinase from this study</th>
<th>Rabbit pyruvate kinase from other studies (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ for ADP</td>
<td>0.4 mm</td>
<td>0.20 mm$^a$ (57)</td>
</tr>
<tr>
<td>$K_m$ for PEP$^b$</td>
<td>0.04 mm</td>
<td>0.048 mm$^a$ (57)</td>
</tr>
<tr>
<td>Maximum velocity at 30°</td>
<td>500-600 μmoles/min/mg</td>
<td>300-400 μmoles/min/mg (57)</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.1</td>
<td>Near 7 (38, 39)</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>230,000</td>
<td>237,000 (40)</td>
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<tr>
<td>Subunit molecular weight</td>
<td>57,000</td>
<td>57,100 (41)</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>4</td>
<td>4 (41)</td>
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<tr>
<td>Number of PEP binding sites</td>
<td>4</td>
<td>4$^c$ (34, 35)</td>
</tr>
<tr>
<td>Partial specific volume</td>
<td>0.740 ml/g</td>
<td>0.74 ml/g (40)</td>
</tr>
<tr>
<td>Extinction coefficient ($E_{280}$)</td>
<td>0.55</td>
<td>0.54 (12)</td>
</tr>
<tr>
<td>Sedimentation coefficient ($s_20, w$)</td>
<td>1.71</td>
<td>1.74 (12)</td>
</tr>
<tr>
<td>Concentration dependence of $E_{280}$</td>
<td>9.94 S</td>
<td>10.04 S$^e$ (40)</td>
</tr>
<tr>
<td>8 × 10$^{-2}$ ml/mg</td>
<td>8.6 × 10$^{-2}$ ml/mg</td>
<td></td>
</tr>
</tbody>
</table>

*a In 0.033 M Tris-HCl, pH 7.4, 0.067 M KCl, 1 mM MgCl$_2$, and 0.5 mM PEP.

*b Phosphoenolpyruvate.

*c In 0.033 M Tris-HCl, pH 7.4, 0.067 M KCl, 2 mM MgCl$_2$, and 1.25 mM ADP.

*d See text for discussion concerning this value.

* A range of 0.55 to 10.17, depending on buffer, has been reported by Wilson et al. (43).

activator of mammalian liver pyruvate kinases, had no effect on the activity of the skeletal muscle isozyme when the latter was assayed as described here.

**DISCUSSION**

The purification procedure described here and summarized in Table I provides bovine muscle pyruvate kinase in good yield and with a high degree of purity. In addition, the procedure seems to work equally well with the rabbit muscle enzyme, and is much simpler than previously published procedures. With slight modifications in buffer concentration and salt gradient, we find that it can also be used to isolate turkey muscle pyruvate kinase. Our preparations do not have the microheterogeneity in isoelectric focusing that Susor et al. (31) reported for commercially obtained crystalline samples of rabbit muscle pyruvate kinase.

Conflicting reports are found in the literature concerning the number of substrate binding sites in rabbit muscle pyruvate kinase. Reynard et al. (32) and Betts and Evans (33) reported the presence of two substrate binding sites in rabbit muscle pyruvate kinase, while Kayne (34) and Cottam and Mildvan...
(35) reported the presence of four substrate binding sites. Most of the experimental difficulties that arise in binding studies, including destruction of substrate during the course of the experiment, result in an erroneously low number of substrate binding sites. We found it necessary in our binding studies to work at 4°C in order to minimize a slow loss of phosphoenolpyruvate during the course of the binding experiment, especially when Mg2+ was present. It is not known at this time whether the loss is catalyzed by pyruvate kinase itself or by trace amounts of an impurity, since we estimate that a phosphatase contaminant of only 0.001% would have been sufficient to cause the observed effect. Because we observe a similar effect with rabbit muscle pyruvate kinase, this problem may well account for the controversy surrounding the number of binding sites in that enzyme. It is likely that both the rabbit and bovine muscle enzymes have four binding sites for phosphoenolpyruvate, or one per subunit.

From the data reported here, it appears that bovine skeletal muscle pyruvate kinase is very similar to the extensively studied rabbit muscle enzyme, although the two are clearly distinguishable by immunological tests. The areas of similarity include kinetic constants, molecular weight, partial specific volume, extinction coefficient, sedimentation coefficient, subunit structure, and the number of binding sites (Table V). In addition, there are strong similarities in amino acid composition (Table II) and, to the extent that we have examined them (Table IV), the cation requirements of bovine muscle pyruvate kinase resemble those reported for the rabbit muscle enzyme (44). In view of these parallel properties, it would be reasonable to take available information on the rabbit muscle enzyme as a starting point for future studies on bovine skeletal muscle pyruvate kinase. Similarly, information obtained from studies on the bovine enzyme should be helpful in understanding rabbit muscle pyruvate kinase and, probably, other mammalian skeletal muscle pyruvate kinases as well.

Acknowledgments—We express our sincere appreciation to the following people: Robert Howard and Robert Becker for the amino acid analyses; Joan Miller for her excellent technical assistance; Bobbie Heussey for the graphics; and John Black, C. J. Chern, B. Straub, and M. Rittenberg of the University of Oregon Medical School for facilities and assistance in performing the immunological tests.

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