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# $M_2$ Isozyme of Pyruvate Kinase from Human Kidney as the Product of a Separate Gene: Its Purification and Characterization†

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ABSTRACT: The $M_2$ isozyme of pyruvate kinase has been purified from human kidney. The procedure involved conventional enzyme purification steps plus an affinity chromatography step utilizing the interaction between the dye, Cibracron blue F3GA, and the pyruvate kinase isozyme. During the purification it was observed that the $M_2$ isozyme is very unstable in the absence of fructose 1,6-bisphosphate. In addition, the electrophoretic mobility of the isozyme in polyacrylamide disc gels at pH 9.3 is greatly affected by the presence or absence of this glycolytic intermediate. The final enzyme product had a specific activity of 127 units/mg of protein and represented a 470-fold purification over the crude extract. The high purity of the enzyme preparation was established by polyacrylamide disc gel electrophoresis in the presence and absence of sodium dodecyl sulfate, by sedimentation velocity and equilibrium analyses, and by $NH_2$-terminal analysis. Characterization of the purified human $M_2$ isozyme showed that it is a tetramer of 206 700 daltons with a sedimentation coefficient ($s_{20,w}$) of 9.25 S. Sodium dodecyl sulfate gel electrophoresis indicated that the isozyme consists of four subunits of very similar or identical molecular weight. $NH_2$-terminal analysis suggested that the peptide chains of the enzyme are blocked. The $M_2$ isozyme cross-reacts with antiserum prepared against the human $M_1$ isozyme. The amino acid composition of the $M_2$ isozyme is distinct from that of the $M_1$ or R isozymes. Based on the amino acid compositions of the purified $M_1$ and $M_2$ isozymes we have concluded that they represent the products of separate genes rather than different molecular forms of the same gene product as others have recently proposed.

The $M_2$ isozyme[1] of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) occurs in human and rat tissues as a major component in kidney and a minor component in liver (Bigley et al., 1968; Imamura and Tanaka, 1972). It is the only pyruvate kinase isozyme found in rat liver cells in culture (Walker and Potter, 1973) and predominates in rat and human hepatomas (Farina et al., 1974; Balinsky et al., 1973a,b), regenerating liver (Bonney et al., 1973; Garnett et al., 1974), and fetal tissue (Imamura and Tanaka, 1972; Balinsky et al., 1973a,b).

From their studies of the pyruvate kinase isozyme distribution in differentiating human tissues Marie et al. (1976) suggested that the $M_1$ and $M_2$ isozymes represent different molecular forms of the same gene product.

We have previously described the purification of the R (Chern et al., 1972) and $M_1$ isozymes (Harkins et al., 1977) of pyruvate kinase from human erythrocytes and skeletal muscle, respectively. In this paper we describe the purification of the $M_2$ isozyme from human kidney and determination of its amino acid composition. This has allowed us to compare the amino acid compositions of all three purified isozymes, $M_1$, $M_2$, and R, and to conclude that $M_1$ and $M_2$ represent the products of separate genes.

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[1] We use the generally accepted L, $M_1$, $M_2$, and R nomenclature in this paper to represent the major pyruvate kinase isozyme present in liver, skeletal muscle, kidney, and erythrocytes, respectively.

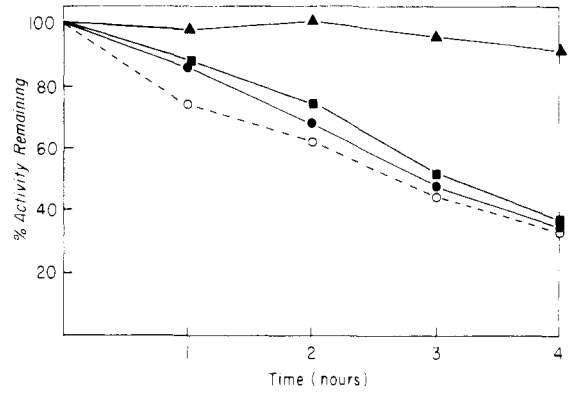


FIGURE 1: Stability of human $M_2$ pyruvate kinase in the presence of substrates and effectors. A 50–65% $(NH_4)_2SO_4$ precipitate of a human kidney extract was dissolved in 25 mM Tris-HCl (pH 7.5), and incubated at 4 °C in the presence of the following effectors: control (○); 1 mM ADP (●); 1 mM phosphoenolpyruvate (■); 0.1 mM fructose 1,6-bisphosphate (▲). The samples were assayed by the standard coupled-assay method. Each point is the average of duplicate assays and is expressed as a percent of the original activity at time zero.

## Experimental Procedure

*Materials.* Chemicals and reagents were purchased from the following sources: the tricyclohexylamine salt of phosphoenolpyruvate, the sodium salt of ADP (grade 1 and fermentation grade), disodium NADH (grade III), the tetrasodium salt of D-fructose 1,6-bisphosphate (grade II and Sigma grade), dansyl chloride,[2] bovine serum albumin, enolase, trypsinogen, ribonuclease, and rabbit muscle lactic dehydrogenase type II containing 1050 IU/mg of protein from Sigma; Ultra Pure urea and ammonium sulfate from Schwarz/Mann; Sephadex G-25 medium, G-200, and Sepharose 6B from Pharmacia; the preswollen, microgranular forms of carboxymethylcellulose (Whatman CM-52) from Reeve-Angel; "Cheng-Chin" polyamide thin-layer sheets from Gallard-Schlesinger; Cibacron blue F3GA from CIBA-Geigy, Chemicals Division, Ardsley, N.Y. All other reagents were the best quality available commercially.

Human kidney was obtained either at autopsy or through the Department of Urology when donated kidneys were unsuitable for transplantation.

Pyruvate kinase was monitored by the coupled assay method of Bücher and Pfleiderer (1955).

*Preparation of Cibacron Blue F3GA–Sepharose 6B Affinity Column.* Two hundred milliliters of preswollen Sepharose 6B was first cross-linked with epichlorohydrin and desulfated by alkaline hydrolysis according to the method of Porath et al. (1971). The Cibacron blue F3GA dye was coupled to the Sepharose by the method of Böhme et al. (1972). The Cibacron blue F3GA–Sepharose 6B used contained 131 μmol of Cibacron blue F3GA bound per g of Sepharose.

*Ultracentrifugation.* Sedimentation velocity studies were performed on the purified enzyme using a Spinco Model E ultracentrifuge. The partial specific volume used in the calculation of the sedimentation coefficient was obtained from the amino acid composition of the purified enzyme by the method of Cohn and Edsall (1943). Before the run the sample was dialyzed for 24 h against the sample buffer, 0.1 M Miller's buffer (pH 7.2) (Miller and Golder, 1950) with 0.1 mM fructose 1,6-bisphosphate.

Sedimentation equilibrium studies were performed by the low-speed method of Chervenka (1969). The molecular weight of the purified human $M_2$ isozyme was determined according to DiCamelli et al. (1970).

*$NH_2$-Terminal Analysis.* Bovine serum albumin and the purified $M_2$ isozyme were dansylated in the presence of 8 M urea according to Gros and Labouesse (1969). The dansylated proteins were hydrolyzed in 6 N HCl for 12 h under vacuum and examined by two-dimensional thin-layer chromatography on 5 × 5 cm polyamide sheets coated on both sides (Hartley, 1970), using water–90% formic acid (100:1.5) in the first dimension, and benzene–glacial acetic acid (9:1) in the second dimension.

*Immunological Studies.* We have previously described the preparation of rabbit antisera to the purified human $M_1$ isozyme (Lincoln et al., 1975; Harkins et al., 1977).

Gel diffusion analyses were carried out on microscope slides (Campbell et al., 1970) in either 0.75% agarose (L'Industrie Biologique Francaise S.A.) or 0.85% ionagar (No. 2, Colab, Glenwood, Ill.) at pH 7.8 in 0.04 M barbital buffer containing 1.0 M glycine and 0.14 M NaCl.

*Amino Acid Analyses.* The amino acid content of the purified $M_2$ isozyme was determined in duplicate after 24-, 48-, and 72-h hydrolysis in 6 N HCl on a Beckman 120 C amino acid analyzer.

## Results

Purification of the $M_2$ isozyme was carried out at 4 °C or with the enzyme solution on ice. During early attempts to fractionate the $M_2$ isozyme, we observed that it was very unstable. At pH 7.5 and 4 °C, the enzyme lost nearly 70% of its original activity within 4 h (Figure 1). However, in the presence of 0.1 mM fructose 1,6-bisphosphate, the enzyme retained 95% of its original activity under the same conditions. Therefore, 0.1 mM fructose 1,6-bisphosphate (Sigma grade) was included in the initial extraction buffer and in all subsequent solutions. The $M_2$ isozyme could be stored at 4 °C as a 75% saturated ammonium sulfate suspension without any significant loss of activity.

*Step 1: Extraction.* Frozen human kidneys were thawed and the adipose tissue removed. The kidneys were minced and mixed with 2 vol of extraction buffer, containing 25 mM Tris-HCl (pH 7.0), 10 mM magnesium sulfate, 1 mM EDTA, 0.1% 2-mercaptoethanol, and 0.1 mM fructose 1,6-bisphosphate. The mixture was homogenized for 2 min in a Waring blender, stirred for 1 h at 4 °C, and then centrifuged for 30 min at 10 000*g*. The supernatant was filtered through two layers of cheesecloth.

*Step 2: Ammonium Sulfate Fractionation.* Solid ammonium sulfate was added slowly with stirring to the extraction supernatant from step 1 to give 50% saturation according to the nomogram of Dawson et al. (1969). The pH of the solution was maintained at 7.0 by dropwise addition of 1 N NaOH. After the salt had been added, the solution was stirred for 15 min and centrifuged at 10 000*g* and the precipitate discarded. The supernatant was placed in an ice bath and taken to 65% saturation with ammonium sulfate.

*Step 3: CM-Cellulose Chromatography.* The 65% ammonium sulfate precipitate from step 2 was collected by centrifugation and dissolved in 33 mM potassium maleate (pH 5.5), containing 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM fructose 1,6-bisphosphate. The sample was desalted on a Sephadex G25 column and applied to a column of CM-cellulose equilibrated with the maleate buffer. Pyruvate kinase was eluted with a pH gradient of 33 mM potassium maleate buffers at pH 5.5 and 7.0. The elution profile is shown in Figure

[2] Abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CM-cellulose, carboxymethylcellulose; OD, optical density.

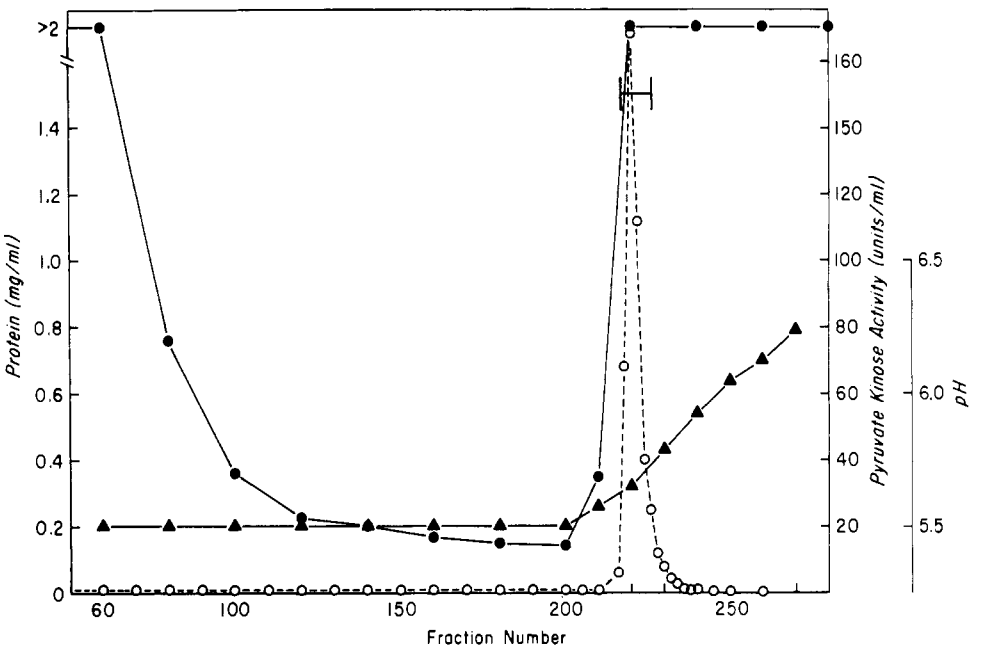


FIGURE 2: CM-cellulose chromatography of the $M_2$ isozyme of pyruvate kinase from human kidney. The product of step 2 was equilibrated with the CM-column buffer, 33 mM potassium maleate (pH 5.5), 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM fructose 1,6-bisphosphate. The sample was applied to a 2.5 × 40 cm column (bed height = 35 cm) of CM-cellulose at a flow rate of 100 mL/h, using a peristaltic pump. The column was washed with CM-column buffer until the effluent fractions contained 200 μg/mL protein or less as judged by optical density at 280 nm, and then a linear pH gradient was applied with 500 mL of starting CM-column buffer (pH 5.5) and 500 mL of starting column buffer titrated to pH 7.0 with 2 M KOH. Ten-milliliter fractions were assayed for pyruvate kinase (O - - - O), protein by the Lowry-phenol method (●—●), and for pH at 4 °C (▲—▲). The fractions 217-227 as indicated by the bar were pooled for subsequent purification.

2. Fractions 217 through 227 were pooled, assayed, and stored at 4 °C as a 75% ammonium sulfate suspension.

*Step 4.* Cibacron blue F3GA-Sepharose 6B affinity chromatography was carried out by a modification of the procedure of Easterday and Easterday (1974). The 75% ammonium sulfate suspension from step 3 was centrifuged and dissolved in affinity column starting buffer consisting of 20 mM Tris-HCl (pH 8.5), 5 mM magnesium chloride, 2 mM 2-mercaptoethanol, 0.4 mM EDTA, 0.3 M potassium chloride, and 0.1 mM fructose 1,6-bisphosphate. The sample was desalted and applied to the affinity column. The column was washed until the effluent fractions had an absorbance at 280 nm of less than 0.2 OD unit and the enzyme was eluted with starting buffer containing 10 mM ADP (fermentation grade). The results are shown in Figure 3. Fractions 88 through 93 were pooled and confirmed to contain the $M_2$ isozyme by thin-layer electrophoresis according to Imamura and Tanaka (1972). After assay the pooled material was stored as a 75% ammonium sulfate suspension. The enzyme which was not absorbed to the column under the conditions used corresponded to the $M_1$ isozyme in electrophoretic mobility.

*Step 5: Sephadex G-200 Chromatography.* The 75% ammonium sulfate suspension from step 4 was centrifuged, dissolved in 25 mM Tris-HCl (pH 7.0), 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM fructose 1,6-bisphosphate (Sigma grade) and applied to a Sephadex G-200 column equilibrated with the same buffer. The elution results are shown in Figure 4. Those fractions (23 through 26) having the highest specific activity, approximately 200 units per OD unit at 280 nm, were pooled and stored as a 75% ammonium sulfate suspension. Fractions 27 through 30 were rechromatographed on the Sephadex G-200 column and those fractions in the second eluate having a specific activity of 200 units per OD unit at 280 nm were combined with the material from the first column. This material was used in the subsequent tests for purity and for characterization.

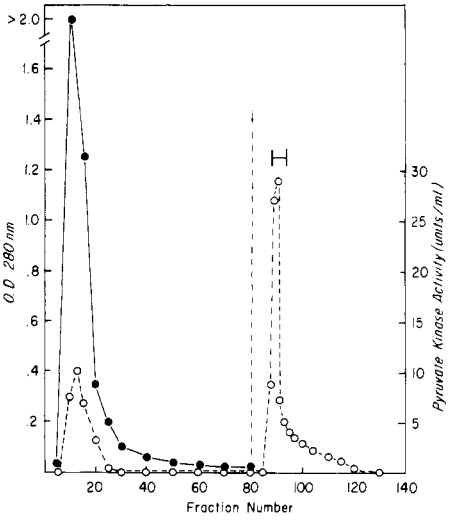


FIGURE 3: Affinity chromatography of the $M_2$ isozyme of pyruvate kinase from human kidney. The enzyme product from step 3 of the purification procedure, stored as a 75% ammonium sulfate suspension, was centrifuged and dissolved in affinity column starting buffer, containing 20 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.4 mM EDTA, 0.1 mM fructose 1,6-bisphosphate, and 0.3 M potassium chloride. The sample was applied to a 2.5 × 40 cm column (bed height, 20 cm) of Cibacron blue F3GA-Sepharose 6B at a flow rate of 100 mL/h. The column was washed with starting buffer until the effluent fractions had an absorbance at 280 nm of 0.2 or less. At the point indicated by the arrow the column was washed with 300 mL of starting buffer containing 10 mM adenosine 5′-diphosphate. Ten-milliliter fractions were assayed for pyruvate kinase (O - - - O) and for absorbance at 280 nm (●—●). Fractions 88-93 were pooled for the next step in the purification.

Table I shows the results of the purification of the human $M_2$ isozyme of pyruvate kinase from 550 g of kidney.

*Criteria of Purity.* Polyacrylamide Gel Electrophoresis. In the absence of fructose 1,6-bisphosphate the product of step 5 migrated as a major component at pH 9.3 with only traces

TABLE I: Purification of Human $M_2$ Pyruvate Kinase Isozyme.

| Purification step | Total units[a] | Yield (%) | Total protein (mg) | Sp act.[b] (units/mg of protein) | Purification |
|---|---|---|---|---|---|
| 1. Crude extract | 23 150 | 100 | 85 740 | 0.27 | 1 |
| 2. 50–65% ammonium sulfate precipitate | 17 370 | 75 | 37 760 | 0.46 | 1.7 |
| 3. CM-cellulose chromatography | 7 646 | 33 | 294 | 26.0 | 96.3 |
| 4. F3GA affinity chromatography: ADP elution | 1 325 | 5.7 | 12.9 | 103.0 | 381.5 |
| 5. Sephadex G-200 chromatography | 849 | 3.7 | 6.7 | 127.0 | 470 |

[a] A unit of pyruvate kinase activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH per min under the conditions for the coupled assay (Bücher and Pfleiderer, 1955). [b] Specific activity is defined as units per milligram of protein, where protein is measured by the modified Lowry–phenol method of Oyama and Eagle (1965).

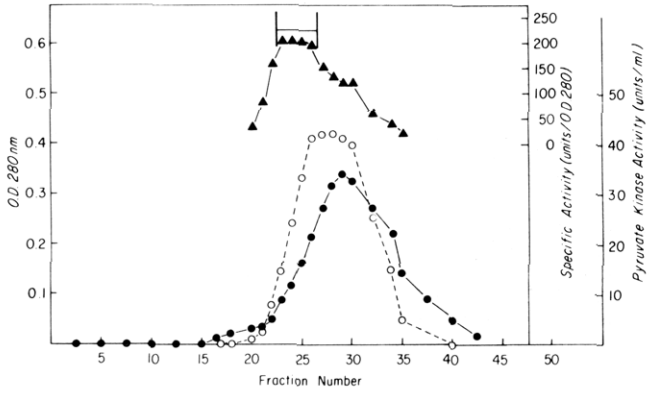


FIGURE 4: Sephadex G-200 chromatography of the $M_2$ isozyme of pyruvate kinase from human kidney. The enzyme product from step 4 (pool 88–93, Figure 3) was concentrated by 75% $(NH_4)_2SO_4$ precipitation, dissolved in 25 mM Tris-HCl (pH 7.0), 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM fructose 1,6-bisphosphate, and applied to a 1.5 × 100 cm column of Sephadex G-200. Fractions of 3.0 mL were assayed for pyruvate kinase (O - - - O) and for absorbance at 280 nm (●—●). The specific activity is expressed as units of pyruvate kinase per optical density at 280 nm (▲—▲). As indicated by the bar, fractions 23–26 were pooled for the subsequent characterization.

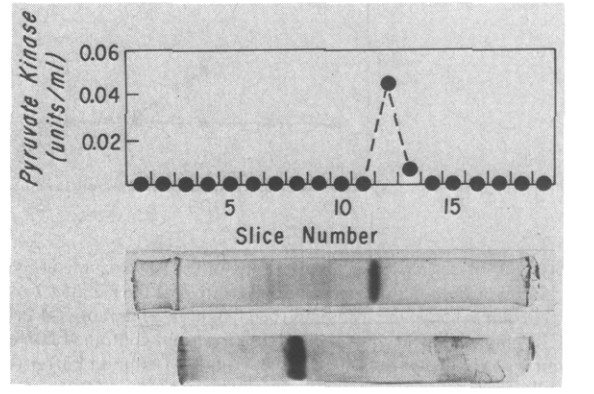


FIGURE 5: Polyacrylamide disc gel electrophoresis of the $M_2$ isozyme of pyruvate kinase from human kidney. The upper gel and figure show the results of polyacrylamide gel electrophoresis in the absence of fructose 1,6-bisphosphate. The upper gel was stained with Amido Schwarz. A duplicate unstained gel was cut into 4-mm sections and each section incubated for 30 min at 25 °C in 500 μL of the upper gel buffer. Pyruvate kinase activity was assayed in 100 μL of the incubation solution. The activity peak in the upper part of the figure coincided with the major protein band. The lower gel shows the result of disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol (Weber and Osborn, 1969).

of contaminants (Figure 5). Immediately after electrophoresis the unstained gel was cut into 4-mm sections and each section incubated for 30 min at 25 °C in 500 μL of upper gel buffer. Then 100 μL of the incubation solution was assayed for pyruvate kinase activity. The results showed that the pyruvate kinase activity coincided with the major protein band in the duplicate stained gel.

In the presence of fructose 1,6-bisphosphate the enzyme had a low mobility on polyacrylamide gel electrophoresis. This observation is consistent with the ligand affecting the state of polymerization of the enzyme as discussed below. Some of the minor bands apparent on polyacrylamide gel electrophoresis are presumably due to higher molecular weight polymers of the enzyme (Figure 5). When the purified preparation was run on polyacrylamide gel electrophoresis in sodium dodecyl sulfate a single component was detected with a very faint trace of a faster migrating component.

The single band obtained with the $M_2$ isozyme on electrophoresis in sodium dodecyl sulfate (Figure 5) suggests that the subunits are very similar or identical in molecular weight. The subunit molecular weight calculation (Weber and Osborn, 1969) gave a value of 50 500 ± 2500 by interpolation of the best fit line through the points plotted for the mobilities of the standard proteins run under the same conditions.

Ultracentrifugation. A single symmetrical peak was obtained on sedimentation velocity analysis consistent with a homogeneous preparation sedimenting with an $s_{20,w}$ of 9.25 S. Sedimentation equilibrium analysis also indicated that the enzyme preparation was pure by the linear relationship between fringe displacement ($\Delta Y$) and the square of the radius ($r^2$). Using the partial specific volume of 0.716 $cm^3/g$ calculated from the amino acid composition, a molecular weight of 206 700 was obtained. The results from polyacrylamide gel electrophoresis are therefore consistent with a tetrameric structure for this isozyme in agreement with previous results for the human R (Peterson et al., 1974) and $M_1$ isozymes (Harkins et al., 1977).

$NH_2$-Terminal Analysis. Ten nanomoles of purified $M_2$ isozyme was dansylated, hydrolyzed, and examined by thin-layer chromatography. No dansylated amino acid was observed other than the expected reaction products of tyrosine, lysine, dansylamide, and dansyl acid. The method was checked using 5 nmol of bovine serum albumin and the $NH_2$-terminal residue could be clearly identified as aspartic. The results suggest that the $NH_2$ terminus of the $M_2$ isozyme is blocked or that it is proline or tryptophan both of which are destroyed by acid hydrolysis. There are no contaminating proteins in the $M_2$ preparation with detectable $NH_2$ terminals within the limits of the method.

Amino Acid Analysis. The amino acid composition of the purified $M_2$ isozyme is shown in Table II. The results are expressed as residues per mole of 206 700 g as determined by the ultracentrifuge studies.

Immunological Studies. The results of immunodiffusion studies using rabbit anti-human $M_1$ pyruvate kinase are shown

TABLE II: Amino Acid Composition of Human Kidney $M_2$ Pyruvate Kinase Isozyme Compared to the Human Muscle and Human Erythrocyte Isozymes.

| | μmol/mg of protein[e] | | | | | | |
|---|---|---|---|---|---|---|---|
| Amino acid | 24 h | 48 h | 72 h | Av value[a] | Residues[b] | $M_1$[c] | R[d] |
| Lys | 0.551 | 0.549 | 0.549 | 0.550 | 114 | 145 | 81 |
| His | 0.211 | 0.210 | 0.205 | 0.208 | 43 | 52 | 43 |
| $NH_3$ | 1.015 | 1.029 | 1.044 | 1.001 | 207 | 180 | 204 |
| Arg | 0.503 | 0.507 | 0.505 | 0.504 | 104 | 120 | 202 |
| Asp | 0.818 | 0.823 | 0.822 | 0.821 | 170 | 199 | 137 |
| Thr | 0.465 | 0.460 | 0.441 | 0.474 | 98 | 111 | 83 |
| Ser | 0.525 | 0.516 | 0.499 | 0.537 | 111 | 120 | 127 |
| Glu | 0.848 | 0.853 | 0.869 | 0.857 | 177 | 237 | 230 |
| Pro | 0.432 | 0.435 | 0.439 | 0.436 | 90 | 87 | 96 |
| Gly | 0.493 | 0.489 | 0.497 | 0.493 | 102 | 165 | 168 |
| Ala | 0.829 | 0.816 | 0.807 | 0.818 | 169 | 218 | 203 |
| Half-Cys[f] | | | | 0.242 | 50 | 61 | 23 |
| Val | 0.713 | 0.721 | 0.722 | 0.722 | 149 | 185 | 187 |
| Met[g] | | | | 0.469 | 97 | 78 | 46 |
| Ile | 0.576 | 0.587 | 0.590 | 0.590 | 122 | 138 | 132 |
| Leu | 0.621 | 0.636 | 0.656 | 0.638 | 132 | 161 | 162 |
| Tyr | 0.148 | 0.142 | 0.145 | 0.145 | 30 | 38 | 30 |
| Phe | 0.280 | 0.281 | 0.283 | 0.282 | 58 | 71 | 59 |
| Trp[h] | | | | 0.049 | 10 | 12 | 31 |

[a] The values for threonine, serine, and ammonia were obtained by extrapolation to zero time. The 72-h values for valine and isoleucine were used. [b] Calculated for a molecular weight of 206 700 obtained by sedimentation equilibrium analyses. [c] Data on the amino acid composition of the human $M_1$ isozyme are from Harkins et al. (1977). [d] Data on the amino acid composition of the human R isozyme are from Chern et al. (1972). [e] The value presented is the average of duplicate analyses performed at each time interval. [f] Determined as cysteic acid following performic acid oxidation (Moore, 1963). [g] Determined as methionine sulfone following performic acid oxidation (Moore, 1963). [h] Determined spectrophotometrically by the method of Goodwin and Morton (1946).

in Figure 6. There is a precipitin line of identity between the human $M_2$ and $M_1$ isozymes with these four antisera indicating that the dominant immunological determinants on the $M_1$ isozyme are also found on the $M_2$ molecule. Both the $M_1$ and $M_2$ isozymes were inactivated by anti-$M_1$ antiserum (Figure 7), reinforcing the conclusion that there are no major antigenic distinctions between them. While the $M_2$ isozyme is inactivated by the anti-$M_1$ antiserum it is not inactivated by anti-R antiserum (Lincoln et al., 1975).

## Discussion

Pyruvate kinase occurs in human kidney as a number of isozymes which can be resolved by thin-layer polyacrylamide gel electrophoresis. These include the L and $M_2$ isozymes and a number of activities of intermediate electrophoretic mobility which probably represent hybrids with varying content of subunits from the parental L and $M_2$ tetramers (Lincoln et al., 1975). By the addition of fructose 1,6-bisphosphate to all solutions during purification and inclusion of a selective affinity chromatography step in the purification scheme we have been able to purify the $M_2$ isozyme from human kidney with a 3.7% yield. The yield is calculated from the total pyruvate kinase activity in a kidney homogenate which will include all isozymes present and underestimate the recovery of the $M_2$ isozyme. By a number of criteria the final product is of high purity.

The pyruvate kinase isozymes differ in their kinetic response to the glycolytic intermediate fructose 1,6-bisphosphate, the product of one of the prior rate-controlling steps in glycolysis. In the absence of L-phenylalanine the kinetics of the $M_1$ isozyme are not affected by fructose 1,6-bisphosphate while the $M_2$ and L isozymes are activated by this intermediate. The rat L isozyme is less stable in the presence of fructose 1,6-bisphosphate (Van Berkel et al., 1975), a labilizing effect which was first reported for the yeast enzyme which is also allosteric (Kuczenski and Suelter, 1970, 1971). In contrast, as we show in Figure 1 the human $M_2$ isozyme is stabilized by fructose 1,6-bisphosphate. Imamura et al. (1972) included fructose 1,6-bisphosphate in all chromatographic solvents in their purification of the $M_2$ isozyme from rat hepatoma cells for reasons they do not explain. Sparmann et al. (1973) observed an effect of fructose 1,6-bisphosphate on the molecular weight of the $M_2$ isozyme from Ehrlich ascites tumor cells. By sucrose density centrifugation they obtained values of 100 000 in the absence and 220 000 in the presence of fructose 1,6-bisphosphate consistent with the effector influencing a dimer–tetramer equilibrium. Spellman and Fottrell (1973) reported a molecular weight of 126 000 for the human $M_2$ isozyme from placenta by Sephadex G200 gel filtration in the absence of fructose 1,6-bisphosphate. The value of 206 700 we report here was obtained in the presence of fructose 1,6-bisphosphate. We have observed a greatly decreased electrophoretic mobility for the $M_2$ isozyme when run in the presence of fructose 1,6-bisphosphate in comparison to the mobility shown in Figure 5 in its absence. The data on the $M_2$ isozyme, including the stability, are consistent with fructose 1,6-bisphosphate influencing a dimer–tetramer equilibrium in favor of the tetramer. This is in contrast to the effect of fructose 1,6-bisphosphate on the yeast enzyme where the dimer–tetramer equilibrium is altered in the direction of the dimer (Kuczenski and Suelter, 1971). Both the yeast and rat L isozyme of pyruvate kinase are made labile by fructose 1,6-bisphosphate while the $M_2$ isozyme is stabilized suggesting that there are fundamental differences in the molecular response of the various isozymes to this organic phosphate even though the kinetic effect of the modulator in each instance is activation. Fructose 1,6-bisphosphate may influence the distribution of L and $M_2$ tetramers and intermediate hybrids in cells where both L and $M_2$ subunits are synthesized.

The physical and chemical properties of the $M_1$, $M_2$, and R isozymes of human pyruvate kinase are compared in Table III. The properties are analogous to those reported for the series of rat pyruvate kinase isozymes by Imamura et al.

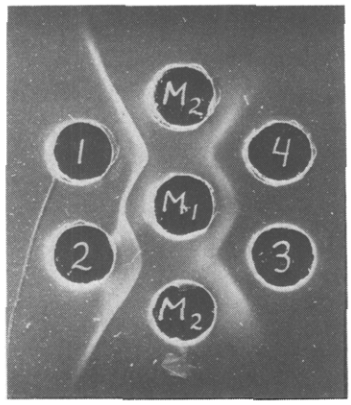


FIGURE 6: Immunological identity of human $M_2$ and $M_1$ pyruvate kinase isozymes. Wells 1, 2, 3, and 4 contained antiserum prepared against human $M_1$ pyruvate kinase. Each well contained antiserum obtained from a different rabbit. In a previous study (Lincoln et al., 1975) we used one of three antisera all prepared identically and at the same time; we use all three in this study (antisera 1, 2, and 3 in Figure 6). In addition, we used a fourth anti-$M_1$ antiserum prepared by immunizing a rabbit with 0.4 mg of $M_1$ complexed with 0.4 mg of methylated bovine serum albumin in 1 mL of complete Freund's adjuvant according to the method of Plescia et al. (1964) as described previously (Rittenberg et al., 1975). The rabbit was boosted with 0.8 mg of $M_1$ and 0.8 mg of methylated bovine serum albumin in 1 mL of complete Freund's adjuvant 4 months after the first injection. The animal was bled at 4 and 5 weeks after the last injection. Sera from the two bleedings were pooled and the globulin fraction obtained by ammonium sulfate fractionation as described previously (Rittenberg et al., 1975). The fraction was passed over a human serum albumin–Sepharose 4B immunoabsorbent column (Fuchs and Sela, 1973) before use. The antisera were obtained after long immunization (10 months in the case of antisera 1, 2, and 3) and presumably recognize many determinants on $M_1$ but do not precipitate or neutralize the R isozyme. The $M_2$ isozyme was obtained from step 3 of the purification procedure and contained 3.6 units/mL. The $M_1$ isozyme was purified from human muscle as described previously (Harkins et al., 1977) and contained 4.0 units/mL. The antigen concentrations chosen were those which were optimal for antiserum number 1; sharp lines to the other antisera could be obtained with different concentrations.

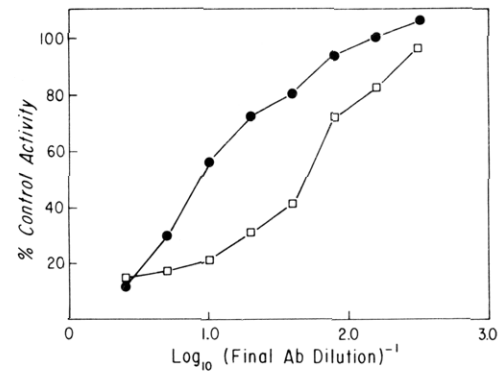


FIGURE 7: Representative data showing inactivation of the human $M_1$ (□) and $M_2$ (●) isozymes of pyruvate kinase by anti-$M_1$ antiserum. The enzyme concentration was adjusted to 0.8 unit/mL. The antiserum was serially diluted with 100 mM triethanolamine–HCl buffer (pH 7.4). Equal volumes of enzyme and diluted antiserum were mixed and incubated at 37 °C for 5 min. At the end of the incubation period a 20-μL aliquot was assayed for enzyme activity in the standard assay. A control containing diluent in place of antiserum was included with each set and inactivation expressed as a percentage of the control value. In a separate experiment normal rabbit antiserum did not inactivate the $M_1$ isozyme.

TABLE III: Properties of the Human Pyruvate Kinase Isozymes.[a]

| | $M_1$ | $M_2$ | R |
|---|---|---|---|
| Sp act. (units/mg) | 382.5 | 127 | 77.4 |
| $S_{0.5}$ phosphoenolpyruvate (mM) | 0.05 | 0.25 | 0.40 |
| $S_{0.5}$ ADP (mM) | 0.45 | 0.30 | 0.25 |
| Mol wt | 240 700 | 206 700 | 225 400 |
| Subunit mol wt | 61 000 | 50 500 | 60 000 |
| $NH_2$ terminal | Blocked | Blocked | Blocked |
| Immunological react. | | | |
| With anti-$M_1$ antiserum | + | + | − |
| With anti-R antiserum | − | − | + |

[a] Data on the $M_1$ isozyme are from Harkins et al. (1977) and the R isozyme from Chern et al. (1972) and Peterson et al. (1974). The $S_{0.5}$ values were obtained at 25 °C and pH 7.2 using the pH stat assay for pyruvate kinase as described by Melchior (1965).

(1972a,b). The human isozymes all have molecular weights in the 200 000–240 000 range. The subunit molecular weights as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicate that the three isozymes are tetramers of 50 000–60 000 molecular weight subunits. We have shown that the subunits of the human $M_1$ isozyme are identical while those of the human R isozyme probably consist of two nonidentical pairs (Peterson et al., 1974). Only one band was detected on sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the $M_2$ isozyme implying that the $M_2$ subunits are identical in size. Additional work is required to establish whether the subunits are also identical in composition. It is of interest that the human L isozyme which has not been characterized physicochemically can be grouped with the R isozyme by immunological cross-reactivity (Table II) in accord with the recent suggestions of Marie et al. (1976).

The three human isozymes all have blocked or labile $NH_2$-terminal amino acids. Similar results have been reported for all other pyruvate kinase molecules which have been studied including Baker's yeast (Yun et al., 1976), Brewer's yeast (Bornmann et al., 1972), and *Escherichia coli* (Waygood and Sanwal, 1974). Cottam et al. (1969) identified *N*-acetyl as the $NH_2$-terminal blocking group on rabbit muscle pyruvate kinase. The extrapolation that this is true for all other pyruvate kinase molecules seems logical but has not been tested.

The human R isozyme is clearly immunologically distinct from the $M_1$ and $M_2$ isozymes by immunodiffusion and enzyme inactivation experiments (Lincoln et al., 1975). The immunological experiments we report here do not distinguish between the $M_1$ and $M_2$ isozymes. Imamura et al. (1972a,b) showed that while the rat $M_1$ and $M_2$ isozymes cross-react there were immunological differences detectable by chicken anti-rat $M_2$ pyruvate kinase antiserum. Our inability to detect such antigenic differences in the human may be due to a specific failure of the particular rabbits we used to recognize $M_1$ specific antigenic determinants although Marie et al. (1976) also reported immunological identity between human $M_1$ and $M_2$ using a rabbit anti-$M_2$ antiserum. Thus, it is more likely that the chicken can more easily detect differences in determinants between $M_1$ and $M_2$ than the rabbit due to the latter's closer evolutionary relationship to man. Clearly, more extensive immunological studies on the relationship of pyruvate kinase isozymes are warranted.

Corcoran et al. (1976) have recently described the purification of a pyruvate kinase isozyme from human lung which they classify as $M_2$ type. Their reported amino acid composition differs from that of human kidney $M_2$ pyruvate kinase shown in Table II. In electrophoretic studies (unpublished) of pyruvate kinase isozyme distribution in human tissues we have observed a number of bands in human lung including the $M_2$ isozyme. It is possible that the isozyme purified by Corcoran et al. is not the $M_2$ isozyme.

The amino acid compositions of the human $M_1$ and $M_2$ isozymes shown in Table II do not support the proposal that

these isozymes represent different molecular forms of the same gene product (Marie et al., 1976). We have analyzed all available pyruvate kinase amino acid compositions (Black and Harkins, 1977) including the $M_1$ and $M_2$ values given in Table II. The five $M_1$ isozymes show greater similarities in composition than do the human $M_1$ and $M_2$ isozymes. The differences between $M_1$ and $M_2$ could possibly be due to proteolytic cleavage of $M_1$ subunits to give the smaller $M_2$ molecule; however, this would be inconsistent with the $M_2$ to $M_1$ transition proposed by Marie et al. (1976), since in that instance we would expect the $M_2$ molecule to be the larger. The evidence is best interpreted on the basis of the evolutionary divergence of $M_1$ and $M_2$ genes before the speciation of mammals. The $M_1$ and $M_2$ isozymes, therefore, represent the products of separate but related genes.

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