Pyruvate Kinase Isozymes in Adult Tissues and Eggs of *Rana Pipiens*.

III. Comparative Studies on the Properties of the Different Isozymes¹

by

L. H. Schloen,² E. H. Kmiotek and H. J. Sallach

Department of Physiological Chemistry, University of Wisconsin Medical School,
Madison, Wisconsin 53706

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Send correspondence to: H. J. Sallach

Department of Physiological Chemistry
683 Medical Science Bldg.
University of Wisconsin Medical School
Madison, Wisconsin 53706

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ABSTRACT

The properties of the isozymes of pyruvate kinase (ATP: pyruvate phosphotransferase, E.C.2.7.1.40) found in unfertilized frog egg have been compared to those found in adult tissues of *Rana pipiens*. Chromatographic, kinetic and electrophoretic data indicate that, of the five electrophoretic forms found in egg, the isozyme with the least anodic mobility (isozyme I) is the same molecular species as the only isozyme found in heart and the egg isozyme with the greatest anodic mobility (isozyme V) is identical to the major isozyme found in liver.

The activity of egg isozyme I was markedly inhibited by the antibody to the skeletal muscle enzyme, which has been shown previously to cross-react with the cardiac enzyme, but was unaffected by the antibody to liver isozyme V; the opposite effects were observed with egg isozyme V. The antibody to the skeletal muscle enzyme inhibited egg isozymes II > III > IV whereas the antibody to the liver enzyme gave the reverse inhibitory pattern, e.g. isozyme IV > III > II.

In vitro dissociation-reassociation of mixtures of isozyme I and V led to the formation of the other three isozymes. Similar experiments performed individually with either egg isozyme III or IV resulted in the production of predominantly isozymes III, II and I due to the instability of isozyme V during the hybridization procedure.

The above results indicate that isozymes I and V are tetramers of the respective parental subunits and that isozymes II, III and IV are hybrid molecules with subunit assignments of (I₃V₁), (I₂V₂) and (I₁V₃), respectively.

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, E.C.2.7.1.40) exists in multiple molecular forms in different tissues (1-16). On the basis of electrophoretic, chromatographic, immunological and kinetic properties, there appear to be at least three major, unique forms of pyruvate kinase in adult tissues of various animals. Using the nomenclature introduced by Tanaka and associates (11), these isozymes have been designated as types M_1 , M_2 and L.

Type L pyruvate kinase is the major isozyme of liver and a minor component of kidney and recent evidence indicates that it is the only isozyme present in liver parenchymal cells (17,18). This isozyme displays cooperative kinetics with respect to PEP^3 and its activity is affected by various metabolites including FDP, ATP, L-alanine and L-phenylalanine (4,8,16,19-27). Type L pyruvate kinase appears to exist in two interconvertible forms differing in their sensitivity to the effects of the above modifiers (21,23-25,27) which may relate to the oxidation state of sulfhydryl groups (27) or to very tight binding of ligands such as FDP (23,25) although organic solvents have been shown to effect the interconversion (24). This isozyme changes under different dietary and hormonal conditions suggesting that it plays an important role in the regulation of glycolysis and gluconeogenesis (2,28).

Type M_1 pyruvate kinase is the only form found in skeletal muscle and is the major isozyme found in mammalian brain and heart (3,4,11-16). It displays Michaelis-Menten kinetics with respect to substrate and is unaffected by FDP and amino acids (3,16,24,29,30) although inhibition by L-phenylalanine has been reported (31,32).

Type M₂ pyruvate kinase has a broad distribution in most adult tissues (4,11-16) and is found in all fetal tissues (12,33,34), in hepatomas (24,35,36) and in regenerating liver (cf. 37). This isozyme exhibits cooperative kinetics with respect to PEP and is affected by allosteric modifiers but important differences exist between its kinetic properties and those of types L and M₁ (9,16,24). Furthermore, it is clearly distinguishable from types M₁ and L on the basis of other properties (cf. 24). Type M₂ pyruvate kinase also appears to exist in two interconvertible forms one of which is highly sensitive to allosteric modifiers and the other less sensitive (5,39).

With respect to immunological properties, no cross-reactivity occurs between types M₁ and L pyruvate kinases but a very significant degree of cross-reactivity has been demonstrated between types M₁ and M₂ (3,4,6,12,40). However, recent studies by Imamura et al. (24), using an antibody prepared against type M₂ from Yoshida ascites hepatoma 130 cells, have shown that types M₁ and M₂ are immunologically distinguishable.

Work from a number of laboratories has established that type M₁ pyruvate kinase from a variety of species has a tetrameric structure (30,40,41) as does type L from either pig (42) or beef (26) liver. *In vitro* hybridization studies (10,43) support the conclusion that intermediate forms of pyruvate kinase found in different tissues represent tetrameric hybrids formed from different parental type subunits.

The developing amphibian is being used in this laboratory as a model system for a study of biochemical differentiation. Comparative studies carried out earlier (7) demonstrated that there are five different electrophoretic forms of pyruvate kinase in the unfertilized frog egg and certain adult tissues; these have been designated as isozymes I through V on the basis

of increasing anodal mobilities. Isozymes I and V from egg correspond in mobility to the only isozyme found in cardiac tissue and to the major component found in liver, respectively. On the other hand, the only isozyme found in skeletal muscle differs in electrophoretic mobility from the other five isozymes. Both isozyme I from heart and isozyme V from liver are allosterically modifiable enzymes whereas the enzyme from skeletal muscle is not (7,40). Although isozyme I from cardiac tissue cross-reacts with the antibody to the skeletal muscle enzyme, the two enzymes are clearly distinguishable on the basis of kinetic, chromatographic and electrophoretic properties (7,40).

In this paper, further studies on the interrelationships between the properties of the isozymes found in various adult and undifferentiated tissues from the grassfrog, *Rana pipiens*, are reported. Kinetic, chromatographic, immunological and other data are presented which strongly support the concept that isozymes I and V of egg are identical to the corresponding isozymes found in heart and liver, respectively, and that the intermediate isozymes found in eggs and certain adult tissues of this species are tetrameric hybrids of these two parental types as originally postulated (7).

MATERIALS AND METHODS

Materials.

PEP, ADP, ATP, FDP, L-alanine, L-phenylalanine and lactate dehydrogenase (E.C.1.1.1.28) were purchased from Sigma. DEAE-, CM- and G-200 Sephadexes were obtained from Pharmacia. Other reagents were commercial preparations of the highest purity available.

Adult male and gravid female grassfrogs, *Rana pipiens*, were purchased from the Mogul-Ed Corp., Oshkosh, Wisconsin. Gravid females that had been induced to ovulate by the injection of a pituitary suspension were obtained in this form from the commercial source. Eggs were stripped 24-48 hrs after injection.

Enzyme Assay.

Pyruvate kinase was assayed by the method of Bücher and Pfeider (44) except that 0.46 mM ADP was employed in the assay system. In this spectrophotometric assay, the pyruvate kinase reaction is coupled with that of lactate dehydrogenase. All kinetic experiments were performed with 25-40 units of pyruvate kinase in the assay system; other conditions were those described previously (7,40). One unit of enzyme activity is defined as that amount of enzyme which catalyzes a change of 0.001 in absorbance at 340 nm per min. Protein was determined by the method of Lowry et al. (45) using bovine serum albumin as the standard.

Enzyme Preparations.

Animals were killed by decapitation and the appropriate tissues removed. The tissue was homogenized in two volumes (w/v) of 0.05 M imidazole acetate

buffer, pH 7.4, containing 0.08 M KCl, 0.01 M 2-mercaptoethanol and 0.001 M EDTA, for 1 min in a Waring blender. The homogenate was centrifuged at $14,000 \times g$ for 30 min and the resulting supernatant solution centrifuged at $105,000 \times g$ for 30 min. The supernatant solution obtained in the latter centrifugation is referred to as the crude tissue extract which, after dialysis against the appropriate buffer, was used for electrophoretic and immunological studies. The two types of egg preparations used in these studies were egg plus ovary from non-induced gravid females and ovulated eggs. The jelly coats of the latter were removed as described previously (46) prior to homogenization and preparation of the crude extract.

In certain experiments, ammonium sulfate precipitates of the above tissue extracts were used. These were prepared at 4° by the slow addition, with stirring, of the calculated amount of solid salt (w/v). Suspensions were equilibrated with stirring for 15 min prior to centrifugation at $14,000 \times g$ for 30 min. For extracts of cardiac tissue, 18 g of ammonium sulfate was added per 100 ml of tissue extract. The 0-18% ammonium sulfate precipitate was removed by centrifugation and ammonium sulfate (20 g/100 ml of original extract) was added to the resulting supernatant solution. The 18-38% ammonium sulfate precipitate was recovered by centrifugation and used. In experiments with egg preparations, 42 g of ammonium sulfate was added to each 100 ml of tissue extract and the 0-42% ammonium sulfate precipitate was recovered by centrifugation and used.

In experiments in which individual isozymes from egg or liver were used, they were prepared as follows. There are five isozymes of pyruvate kinase in liver with isozyme V as the major component. The latter isozyme has been separated from the other four and brought to a high state of purity by

procedures described elsewhere (47). Disc gel electrophoresis of this fraction by the method of Ornstein and Davis (48) at varying protein concentrations revealed one major protein band and a very faint minor band which did not correspond in electrophoretic mobility to any of the five isozymes. All pyruvate kinase activity was associated with the major band when gels were stained for enzyme activity. This fraction was used in all experiments which refer to liver isozyme V; it was also used for the preparation of antisera as described below.

The chromatographic separation of the five egg isozymes is described in the section below.

Ion-Exchange Chromatography.

Before their use, Sephadex resins were treated according to the directions supplied by Pharmacia. In column chromatographic procedures, gels were equilibrated with the appropriate buffer prior to the pouring of the columns which were then washed with several column volumes of buffer before use. All gradients were made by dissolving KCl in the column buffer. Protein in column eluates was detected by absorbance at 280 nm. Enzyme solutions were concentrated by ultrafiltration (Diaflo ultrafiltration cell, Amicon Corp.).

For the separation of the five isozymes found in egg, the 0-42% ammonium sulfate precipitate of the tissue extract (130 mg of protein) was dissolved in, and dialyzed against, 0.09 M imidazole acetate buffer, pH 7.4, containing 0.01 M 2-mercaptoethanol, 0.001 M EDTA and 0.5 mM FDP, until freed of ammonium ions. The enzyme solution was then applied to a column (2.4 × 20 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was washed with 200 ml of application buffer and then eluted with a 0-0.15 M KCl gradient. The

mixing vessel contained 450 ml of the above buffer and the reservoir contained 450 ml of 0.15 M KCl dissolved in buffer. Fraction volume was 9 ml. Fractions comprising the individual peaks of pyruvate kinase activity were pooled and concentrated to approximately 1 mg of protein per ml. Pyruvate kinase was precipitated by the addition of 60 g of ammonium sulfate per 100 ml of solution and the 0-60% ammonium sulfate precipitates retained. In all cases, isozymic purity was confirmed by electrophoresis as described below. If the isozymes were not completely separated in the initial chromatography, they were re-chromatographed under identical conditions.

As discussed in Results, two kinetically different forms of either isozyme I or isozyme V are produced upon CM-Sephadex chromatography. The general chromatographic conditions are outlined here and other experimental conditions are detailed in the legends to the appropriate figures. For isozyme I, the enzyme was dissolved in, and dialyzed against, 0.04 M imidazole acetate buffer, pH 7.4, containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA, until free of ammonium ions. The enzyme solution was then applied to a column (1.8 × 40 cm) of CM-Sephadex equilibrated with the same buffer. The column was washed with 50 ml of application buffer and then eluted with a 0-0.15 M KCl gradient. The mixing vessel contained 150 ml of the above buffer and the reservoir contained 150 ml of 0.15 M KCl in the same buffer. Fraction volume was 2.5 ml. After termination of the gradient, column buffer with 0.15 M KCl was used to wash the column until all activity was eluted. Fractions of the individual peaks were pooled and concentrated by ultrafiltration prior to kinetic and electrophoretic analyses. The same general procedure was used for isozyme V with the following modifications. The buffer used was 0.01 M imidazole acetate, pH 6.0, containing 0.01 M 2-mercaptoethanol and 0.001 M

EDTA. The column (2.6 × 50 cm) was washed with 75 ml of application buffer prior to the initiation of a 0-0.2 M KCl gradient (300 ml of solution per chamber). Fraction volume was 5 ml.

Immunological Studies.

The antibody to purified frog skeletal muscle pyruvate kinase was the preparation used in earlier studies (40). Antibody production via injection of purified frog liver pyruvate kinase (isozyme V) into rabbits and the partial purification of this antibody were also carried out as described by Levy and Sober (49). Antisera, collected after injection of a total of 6.0 mg of purified enzyme over a 5 week period, gave a positive interfacial ring test (50) with purified liver pyruvate kinase at a concentration of 0.1 µg per ml. The partially purified antibody was diluted 1:5 in 0.05 M Hepes buffer, pH 7.4, and stored at -15° prior to use. The studies on the effects of the antibodies on the activities of the different pyruvate kinase isozymes were carried out in the following manner. The same amount of isozyme, based on units of activity (25 units), was mixed with increasing volumes of antibody. The antibody, enzyme and other components of the standard assay system, with and without 0.5 mM FDP, were preincubated for 1 min at room temperature prior to initiation of the reaction by the addition of substrate, PEP. Partially purified serum from nonimmunized rabbits was used in place of the antibody in control experiments.

Other Procedures.

The dissociation-reassociation of isozymes for the *in vitro* formation of hybrid molecules of pyruvate kinase was carried out as described by Cardenas and Dyson (26). Electrophoresis on cellulose acetate strips (Sephraphore III,

2.5 × 17 cm, Gelman Instrument Co.) was performed in 0.05 M imidazole acetate buffer, pH 7.4, containing 0.25 M sucrose, 0.5 mM FDP and 0.001 M EDTA.

Electrophoretograms were run at 30 volts per cm for 3 to 4 hours at 4°. Bands of pyruvate kinase activity were visualized as described previously (7).

RESULTS

Electrophoretic and Chromatographic Studies.

The isozymic patterns of pyruvate kinase activity found when crude extracts of different tissues were subjected to electrophoresis and enzymatic staining are shown in Fig. 1. Five bands of activity are found in ovulated egg, egg + ovary and liver whereas only one is observed in heart. On the basis of electrophoretic mobility, these isozymes have been numbered I through V consecutively, I being localized at the most cathodic site and V at the most anodic site (see Fig. 1). Isozymes I and V from egg preparations correspond in mobility to the only isozyme found in heart and to the major component found in liver, respectively. In view of the fact that ovulated egg and egg + ovary preparations give similar patterns and, since the latter are more readily available, egg + ovary was used routinely for the isolation of the individual isozymes by chromatography on DEAE-Sephadex (Fig. 2). In addition to having the same electrophoretic mobilities as the corresponding isozymes from egg, isozyme I from heart and isozyme V from liver have the same elution profiles as their counterparts in egg when each is subjected to DEAE-Sephadex chromatography under identical conditions (Fig. 2).

In earlier studies (40), it was found that chromatography of isozyme I from heart on CM-Sephadex gave two peaks of pyruvate kinase activity. Although the pyruvate kinase in the two fractions had identical electrophoretic mobilities and immunological properties, marked differences in kinetic properties were observed. Hence, it was of interest to determine if isozyme I from egg behaved in a similar manner. Chromatography of isozyme I from either source under identical conditions resulted in two peaks of

pyruvate kinase activity (P-I and P-II) with similar elution profiles (Fig. 3). In addition, the properties of the pyruvate kinase in the two separated peaks (fractions P-I and P-II) from egg differ from each other kinetically as do their counterparts from heart (Table I). A much greater activation by FDP and inhibition by amino acids is observed with the pyruvate kinase in fraction P-II from either source than is obtained with that in fraction P-I. Furthermore, when PEP saturation curves for the different pyruvate kinase fractions are compared (data not shown), pyruvate kinase in fraction P-II from either heart or egg displays sigmoidal kinetics whereas that in fraction P-I gives hyperbolic curves. All fractions give the same level of activity in the presence of 0.5 mM FDP and hyperbolic kinetics. The apparent K_m for PEP found for the pyruvate kinase in the P-II fractions from either source in the absence of FDP (1.9×10^{-4} M) is lowered in the presence of 0.5 mM FDP (0.45×10^{-5} M). There was no difference in the electrophoretic mobility of the pyruvate kinase in the different fractions.

In view of the above results, a similar study was performed to determine if isozyme V from either liver or egg would be affected correspondingly by chromatography on CM-Sephadex. Since isozyme V is not retained on CM-Sephadex under the conditions used for isozyme I, these experiments were carried out at a lower pH and ionic strength. When isozyme V from either liver or egg is applied to a CM-Sephadex column previously equilibrated with 0.01 M imidazole acetate buffer, pH 6.0, and is eluted with a 0-0.2 M KCl gradient, two peaks of pyruvate kinase activity are obtained (Fig. 4); the first one (P-I) is not adsorbed to the resin and appears in the wash before the gradient is applied whereas the second one (P-II) is retained. Upon kinetic analyses of the pyruvate kinase in these fractions (Table I), it was

found that the enzyme in the P-I fraction from either source is the more sensitive form, e.g. it displays greater activation with FDP and more inhibition by amino acids, than was observed with the pyruvate kinase in the P-II fractions. PEP saturation curves (data not shown) for the enzyme in the P-I fractions are sigmoidal whereas those for the pyruvate kinase in the P-II fractions are hyperbolic. The apparent K_m for PEP observed with the pyruvate kinase in the P-I fractions in the absence of FDP (1.3×10^{-4} M) is lowered in the presence of 0.5 mM FDP (1.6×10^{-5} M). Pyruvate kinase in fractions P-I and P-II had identical electrophoretic mobilities.

It is thus apparent, under the chromatographic conditions used, that in the case of isozyme V from egg or liver the first peak of pyruvate kinase (P-I) is the more kinetically sensitive form in contrast to the results obtained with isozyme I from either heart or egg where it was the second fraction (P-II). To determine if these results were due only to the different chromatographic conditions used, or to intrinsic differences between the two isozymes, isozyme I from heart was chromatographed under identical conditions to those used for isozyme V. The results obtained in this control experiment (data not shown) demonstrated that isozyme I retained its original chromatographic properties, e.g. all activity was adsorbed to the resin and the two peaks of enzyme activity were eluted at salt concentrations comparable to those found previously (see Fig. 3). Also, the fraction eluting at the higher salt concentration (P-II) was once again the more sensitive kinetic form.

Several investigators have reported that a given type of mammalian pyruvate kinase, e.g. type M_2 or L, can exist in two different, interconvertible forms which vary in their response to the effects of different allosteric modifiers (21,23-25,27) as found here for isozymes I and V. Evidence has been

presented that this may be due to the very tight binding of effectors such as FDP (23,25). Whether this is the basis for the occurrence of sensitive and less sensitive forms of isozymes I and V observed in the present studies remains to be established. However, since the primary purpose of this work was to compare the properties of egg isozymes with those found in adult tissues, the chromatographic and kinetic data obtained provide further evidence that isozymes I and V from egg are identical to the corresponding isozymes found in heart and liver, respectively.

The kinetic properties of frog skeletal muscle pyruvate kinase established earlier (40) are given in Table I for comparative purposes and differ markedly from those of both isozymes I and V.

Immunological Studies.

In earlier work (40), it was shown that the antibody to the frog skeletal muscle pyruvate kinase cross-reacts with the cardiac enzyme. In the course of these investigations, the effects of this antibody upon the activity of the isozymes from egg and adult tissues were determined in order to gain further understanding of their structural interrelationships. The effects of the antibody to the skeletal muscle enzyme on the activity of the resolved isozymes from egg are shown in Fig. 5. The antibody almost completely inhibited the activity of isozyme I and had a decreasing effect on isozymes II through IV (II > III > IV). The antibody had no effect on the activity of egg isozyme V and similar results were obtained with isozyme V purified from liver. The inhibition curve obtained for the cardiac isozyme was the same as that observed with egg isozyme I.

Rabbit antibody prepared against the purified frog liver enzyme (isozyme V), as described in Materials and Methods, was employed in an analogous fashion in a study of its inhibitory effects on the activity of the various isozymes of pyruvate kinase. The results obtained with the separated isozymes from egg (Fig. 6) were the reverse of those observed with the antibody to the skeletal muscle enzyme, e.g. the greatest inhibition was observed with isozyme V, progressively less inhibition was found for isozymes IV through II, and no inhibition was observed with isozyme I. The inhibition patterns obtained with the antibody to the liver enzyme on crude extracts of heart and on purified liver isozyme V were the same as those found for isozymes I and V from egg, respectively.

In control experiments, it was established that the relative inhibition by either antibody of a particular isozyme was the same regardless of the sensitivity state of the isozyme, as obtained following chromatography on CM-Sephadex, or whether FDP was present in the assay system or absent.

The effects of the two antibodies on pyruvate kinase activity in crude extracts from various tissues are summarized in Table II. Based on the known extent of inhibition of the individual isozymes from egg, the maximal inhibition observed corresponds predictably to the relative amounts of the cross-reacting isozymes present as observed qualitatively on electrophoretograms of tissue extracts (cf. 7). Thus, lung and kidney, which contain predominantly isozymes II and III, are inhibited to a greater extent by the antibody to the skeletal muscle enzyme than by the liver antibody whereas the reverse is true for crude extracts of liver which contains all five isozymes with isozyme V being the major component. The lack of any inhibition of pyruvate kinase

activity in crude extracts of heart and brain by the antibody to purified liver isozyme V is in agreement with the observation that these tissues contain only isozyme I.

In Vitro Hybridization Studies.

It was established in previous work (40) that frog skeletal muscle and cardiac pyruvate kinases have similar molecular weights and that the skeletal muscle enzyme has a tetrameric structure. When the five unresolved isozymes from egg are subjected to gel filtration on G-200 Sephadex columns under the same conditions (40), all five isozymes are eluted as a single peak of activity with an elution volume identical to that found for the heart and muscle enzymes.⁴ Similar results have been obtained with crude liver preparations containing all five isozymes.⁴ These results indicate that isozymes I to V from egg or liver have the same molecular weights as the cardiac and skeletal muscle enzymes and, in view of the tetrameric structure of frog skeletal muscle pyruvate kinase, they are also presumed to be tetramers. If such is the case, then dissociation-reassociation of mixtures of isozymes I and V should lead to the random generation of the three intermediate forms, e.g. isozymes II to IV.

Dissociation-reassociation of isozyme I from heart, using the procedure of Cardenas and Dyson (26), gave approximately 50% recovery of enzyme activity and a single electrophoretic band having the same mobility as the native enzyme (Fig. 7, B). The much greater instability of isozyme V led to technical difficulties. Although there was no change in electrophoretic mobility following denaturation and renaturation of isozyme V from liver (Fig. 7, A), only 12-14% of the original activity was recovered. All attempts to improve

the recoveries by altering the experimental conditions used were unsuccessful. When equal amounts (based on units of activity) of isozyme I from heart and isozyme V from liver were denatured and renatured together, electrophoretic analyses revealed predominantly isozymes I and II with a small amount of isozyme III (Fig. 7, C). In view of the low recoveries with isozyme V, as compared to isozyme I, in control experiments, the level of isozyme V was increased five-fold over that of isozyme I in subsequent dissociation-reassociation experiments to compensate for the lability of the former isozyme. Under these conditions, the electrophoretogram (Fig. 7, D) revealed predominantly isozymes III and IV with lesser amounts of II and V, with only a trace of isozyme I (visible on cellulose acetate strip or upon over-exposure of film). Comparable results were obtained when egg isozymes I and V were subjected to the same procedure. Dissociation-reassociation experiments with isozymes III and IV from egg also resulted in a shifting of the isozyme patterns toward the more stable forms (Fig. 7, F and H, respectively). In fact, the pattern obtained after dissociation-reassociation of egg isozyme III closely resembles that found when equal amounts of isozymes I from heart and V from liver were subjected to this procedure (Fig. 7, C vs. F) as might be expected if egg isozyme III is a tetramer composed of equal numbers of subunits of the parental cardiac and liver types.

DISCUSSION

A primary purpose of the present study was to elucidate the relationships between the isozymes of pyruvate kinase found in adult frog tissues and those present in the unfertilized egg. Earlier electrophoretic studies (7) demonstrated that, of the five isozymes in egg, isozymes I and V corresponded in electrophoretic mobilities to the only isozyme found in heart and to the main isozyme in liver, respectively. As discussed below, the results of the present work confirm the identity of isozyme I from egg with heart as well as that of isozyme V from egg with liver.

Identical elution profiles were obtained for isozyme I from egg and from heart upon chromatography on both DEAE- and CM-Sephadexes. The kinetic properties of isozyme I from either source were also similar. Likewise, isozyme V from either egg or liver had identical chromatographic properties but these differed significantly from those found for isozyme I from either source. The kinetic properties of isozyme V from egg and liver were also similar to each other. The primary kinetic differences between amphibian isozymes I and V are that the former are inhibited to a greater extent by the amino acids, L-alanine and L-phenylalanine. They are both comparably cooperative with respect to PEP, are activated similarly by FDP and can be isolated in more and less sensitive forms with respect to these modifiers upon chromatography on CM-Sephadex.

Further confirmation that egg isozymes I and V are similar to the analogous isozymes in heart and liver, respectively, was obtained in the immunological studies using the antibodies to frog skeletal muscle pyruvate kinase and to the purified liver isozyme V. The activities of isozyme I from

egg and from heart were inhibited in an identical manner, and to the greatest extent, by the antibody to the skeletal muscle enzyme and neither was inhibited by the antibody to the liver enzyme. Reverse effects were observed with isozyme V from both egg and liver. The results of these kinetic, chromatographic, electrophoretic and immunological studies demonstrate that isozymes I and V from egg are identical to their counterparts found in adult tissues.

The results of the immunological studies also establish that isozymes I and V are antigenically different. The fact that egg isozymes II, III and IV are inhibited by both antibodies is indicative that they contain antigenic structures common to both isozymes I and V. Isozyme III is inhibited to approximately the same extent by both antibodies. On the other hand, isozyme II is inhibited to a much greater extent by the antibody to the skeletal muscle enzyme than by that to liver isozyme V whereas the reverse is true for isozyme IV. In fact, the inhibition patterns obtained with the two antibodies for isozymes II, III and IV are similar to those demonstrated in comparable studies with intermediate isozymic forms of lactate dehydrogenase (51) and aldolase (52) which are known to be tetrameric hybrids containing two different types of parental subunits. The immunological data obtained in the present studies are consistent with the fact that isozymes I and V are tetramers containing only one type of subunit and that isozymes II, III and IV have subunit compositions of (I_3V_1) , (I_2V_2) and (I_1V_3) , respectively.

The *in vitro* hybridization experiments provide additional support for the tetrameric structures proposed above for the different isozymes of pyruvate kinase. Dissociation-reassociation of mixtures of isozymes I and V led to the formation of the intermediate electrophoretic forms, e.g. isozymes II, III and

IV, which was not observed when the individual isozymes were subjected to the same procedure separately. The denaturation-renaturation of egg isozyme III led to the formation of isozymes II and I and similar experiments with egg isozyme IV led to the formation of isozymes III, II and I. The failure to generate all five isozymes from a single isozyme can be attributed to the lability of isozyme V that was observed in control experiments with this isozyme. In their studies on the artificial hybrids of beef skeletal muscle and liver pyruvate kinases, which do not occur naturally, Dyson and Cardenas (43) also observed a shifting of the rehybridization patterns toward the muscle forms due to the lability of the liver enzyme.

Earlier studies (40) established that the molecular weights of frog skeletal muscle and cardiac pyruvate kinases are similar and that the skeletal muscle enzyme is a tetramer. Gel filtration studies⁴ indicate that isozymes I through V from egg and liver have similar molecular weights corresponding to that of the skeletal muscle and cardiac enzymes. This observation, together with the results of the immunological and hybridization experiments, as well as the equally spaced electrophoretic mobilities observed for isozymes I through V, would seem to leave little or no doubt that isozymes I-V from egg and liver are tetramers with subunits compositions of (I_4) , (I_3V_1) , (I_2V_2) , (I_1V_3) , and (V_4) , respectively. Furthermore, on the basis of similar electrophoretic mobilities (7) and the immunological results obtained with tissue extracts in the present work, it would appear that the multiple forms of pyruvate kinase observed in other adult amphibian tissues, e.g. kidney, lung, spleen and testes, are also tetramers of the parental isozymes I and V and their hybrids.

The results of previous (7,40) and present work have shown that skeletal muscle pyruvate kinase differs in electrophoretic, chromatographic and kinetic properties from both isozymes I and V. It is similar only immunologically to isozyme I while being unique in every other criterion investigated. Hence, there are three main types of pyruvate kinase in adult amphibian tissues as have been demonstrated in mammalian tissues. A comparison of the properties of the different types of mammalian pyruvate kinases, which were reviewed in the Introduction to this paper, with those established to date for the three main amphibian types indicates that frog skeletal muscle pyruvate kinase, isozyme I and isozyme V have properties similar to those of mammalian types M_1 , M_2 and L, respectively. However, there are differences in the distribution of the types between mammalian (13-16,24) and amphibian systems. For example, type M_1 is the only isozyme found in mammalian heart and the major isozyme in mammalian brain whereas isozyme I (type M_2) is found in the corresponding amphibian tissues. In mammals, type M_2 is the predominant form of pyruvate kinase in fetal (12,33,34) and malignant (24,35,36) tissues. Although it has been shown that, in adult mammalian liver, type M_2 occurs in Kupffer cells whereas type L is found only in parenchymal cells (18,19), recent evidence indicates that parenchymal cells from regenerating liver carry out the synthesis of type M_2 (cf. 37). Hence, type M_2 is considered to be a less specialized form of pyruvate kinase than the other two types in view of its occurrence in fetal and dedifferentiated cancer cells (cf. 24). In contrast, in the amphibian, both isozymes I and V, together with their hybrids, are present in the unfertilized frog egg; hence, in this undifferentiated amphibian tissue, both type M_2 (isozyme I) and type L (isozyme V) are present and occur in a single cell type.

The presence of allosterically modifiable isozymes in unfertilized eggs of *Rana pipiens* indicates that this undifferentiated tissue possesses the ability to respond to metabolic variations between gluconeogenesis and glycolysis. It has been suggested that, in mammalian liver, type L is associated with specialized liver function, e.g. gluconeogenesis, and is involved in the metabolism of the whole body whereas type M₂ is suggested to be involved in supplying basal energy for the maintenance of the liver itself (cf. 24). Whether the different types of pyruvate kinase found in the unfertilized egg serve different functional roles during development remains to be established.

FOOTNOTES

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²Present address: Biomedical Communication and Medical Education, Sloan-Kettering Institute for Cancer Research, New York, New York 10021.

³Abbreviations used are: PEP, phospho-*enol*-pyruvate; FDP, fructose-1,6-diphosphate, Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

⁴L. H. Schloen and H. J. Sallach, unpublished observations.

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Table I

Comparative Studies on the Kinetic Properties of Various Pyruvate Kinase Isozymes Following Chromatography on CM-Sephadex.^a

Enzyme Preparation	Activation with FDP ^b	Percent Control Activity in the Presence of:	
		L-Alanine	L-Phenylalanine
Isozyme I from:			
Heart, CM-Sephadex Fraction P-I ^c	1.0	68	44
" " Fraction P-II ^c	10.6	13	14
Egg, CM-Sephadex Fraction P-I ^c	1.2	55	55
" " Fraction P-II ^c	7.7	25	15
Isozyme V from:			
Liver, CM-Sephadex Fraction P-I ^d	7.3	25	86
" " Fraction P-II ^d	2.0	58	98
Egg, CM-Sephadex Fraction P-I ^d	7.2	23	89
" " Fraction P-II ^d	1.6	63	100
Skeletal Muscle Enzyme ^e	1.0	100	100

^aActivation with FDP and inhibition with L-alanine were performed at 0.034 mM PEP whereas 0.78 mM concentrations were used in the inhibition studies with L-phenylalanine. The levels of FDP, L-alanine and L-phenylalanine were 0.5 mM. For the inhibition studies, the amino acids were added to the complete pyruvate kinase assay system described in Materials and Methods except for PEP which was added 1 min later. Preincubation time with FDP was also 1 min. Other experimental conditions are given in Materials and Methods.

Table I

Footnotes (cont.)

^bThe ratio of activity with FDP to that without FDP.

^cSee Fig. 3.

^dSee Fig. 4.

^eData from Flanders et al. (40) included for comparative purposes.

Table II

Inhibition of Pyruvate Kinase Activity in Tissue Extracts by Antibodies to
Frog Skeletal Muscle Enzyme and to Purified Liver Isozyme V.^a

Tissue Extract	Percent maximal inhibition by the antibody to:	
	Skeletal Muscle Enzyme	Liver Isozyme V
Skeletal muscle	90	0
Heart	90	0
Brain	91	0
Liver	17	62
Kidney	53	38
Lung	57	29
Adipose	66	26
Testes	81	9

^aThe same amount of pyruvate kinase activity (30 units) was incubated with increasing amounts of each antibody under the conditions described in the legend to Fig. 5.

LEGENDS TO FIGURES

Fig. 1. Electrophoretic patterns of pyruvate kinase activities in crude extracts of ovulated eggs (A), egg + ovary (B), liver (C) and heart (D) from *R. pipiens*. Conditions used for the preparation of tissue extracts, electrophoresis and activity staining are described under Materials and Methods. For reference purposes, Roman numerals I through V have been assigned to the isozymes on the basis of their relative electrophoretic mobilities.

Fig. 2. Separation of isozymes of pyruvate kinase by chromatography on DEAE-Sephadex. (●-●, egg; o-o, liver isozyme V; x-x, isozyme I from heart). Enzyme preparations used in the three separate experiments were: egg = 0-42% ammonium sulfate precipitate of tissue extract (270,000 units of activity, specific activity = 2000); liver = purified liver isozyme V (25,000 units of activity, specific activity = 2.2×10^6); and heart = 18-38% ammonium sulfate precipitate (33,000 units of activity, specific activity = 1×10^4). Other experimental details are given in Materials and Methods.

Fig. 3. Elution patterns for pyruvate kinase isozyme I from heart (o-o) and from egg (●-●) when chromatographed separately on CM-Sephadex under identical conditions. Enzyme preparations used in the two individual experiments were: heart = 18-38% ammonium sulfate precipitate (50,000 units of activity, specific activity = 1.0×10^4); and egg = 0-60% ammonium sulfate precipitate of isozyme I isolated by chromatography on DEAE-Sephadex (50,000 units of activity, specific activity = 5.4×10^4). Columns were equilibrated with 0.04 imidazole acetate buffer, pH 7.4, containing 0.01 M 2-mercaptoethanol

and 0.001 M EDTA, prior to application of enzyme; other experimental details are given in Materials and Methods.

Fig. 4. Elution patterns for pyruvate kinase isozyme V from liver (o-o) and from egg (●-●) when chromatographed separately on CM-Sephadex under identical conditions. Enzyme preparations used in the two individual experiments were: liver = purified liver isozyme V, 300,000 units of activity, specific activity = 2.2×10^6); and egg = 0-60% ammonium sulfate precipitate of isozyme V isolated by chromatography on DEAE-Sephadex (91,000 units of activity, specific activity = 7.2×10^4). Columns were equilibrated with 0.01 M imidazole acetate buffer, pH 6.0, containing 0.01 M 2-mercaptoethanol and 0.001 M EDTA, prior to application of enzyme; other experimental conditions are given in Materials and Methods.

Fig. 5. Effect of antibody to frog skeletal muscle pyruvate kinase on the activities of the resolved pyruvate kinase isozymes from frog egg. The same amount of each isozyme, based on units of activity (25 units) was mixed with increasing volumes of antibody. The antibody, enzyme and other components of the standard assay system described in Materials and Methods were preincubated for 1 min at room temperature before the initiation of the reaction with substrate, phospho-*enol*-pyruvate. Partially purified serum from nonimmunized rabbits was used in place of the antibody in control experiments. I through V = egg isozymes separated by chromatography on DEAE-Sephadex.

Fig. 6. Effect of antibody to purified frog liver pyruvate kinase (isozyme V) on the activities of the resolved pyruvate kinase isozymes from frog egg. Experimental conditions were those described in the legend to Fig. 5.

Fig. 7. Electrophoretic patterns of pyruvate kinase isozymes obtained in dissociation-reassociation experiments. After dissociation-reassociation of: (A) purified liver isozyme V alone; (B) cardiac isozyme I alone (18-38% ammonium sulfate fraction); (C) a 1:1 mixture (based on units of activity) of cardiac isozyme I and liver isozyme V; and (D) a 1:5 mixture respectively of cardiac isozyme I and liver isozyme V. Egg isozyme III before (E) and after (F) dissociation-reassociation. Egg isozyme IV before (G) and after (H) dissociation-reassociation. Experimental conditions are described in Materials and Methods.

Fig 1

Fig. 2

Fig. 4

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Fig 6