

AN ABSTRACT OF THE THESIS OF

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Title: PYRUVATE KINASE ISOZYMES IN BEEF AND CHICKEN

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At least three electrophoretically distinguishable, noninterconvertible isozymes of the tetrameric enzyme pyruvate kinase (E. C. 2. 7. 1. 40) are known to exist in mammalian tissues. Type K (or  $K_4$ ) is found in all early fetal tissues and in significant quantities in all adult organs or tissues examined except striated muscle. Type M (or  $M_4$ ) is found mainly in striated muscle and brain. Type L (or  $L_4$ ) is found in mammalian liver, kidney, intestines, and probably erythrocytes. Type L appears to be associated with the major gluconeogenic tissues and has kinetic properties that would appear to adapt it specifically for this purpose.

Equilibrium binding studies of the substrate phosphoenolpyruvate to the bovine skeletal muscle pyruvate kinase ( $M_4$ ) have been carried out using gel filtration and ultracentrifugation. The results are consistent with the presence of four binding sites for phosphoenolpyruvate on the tetrameric pyruvate kinase--i. e., one per

subunit--in contradiction of some earlier studies showing only two binding sites for phosphoenolpyruvate per tetramer. A major difficulty encountered during the equilibrium binding studies was the slow hydrolysis of phosphoenolpyruvate to pyruvate catalyzed by the pyruvate kinase preparations in the absence of ADP. The hydrolysis was greatly reduced by performing the binding studies at 4° instead of room temperature and substituting EDTA for  $\text{MgCl}_2$ . The hydrolysis of phosphoenolpyruvate at 25° in the presence of  $\text{MgCl}_2$  had a specific activity of  $2.3 \times 10^{-4}$  micromoles/min/mg, compared to a specific activity of  $2.3 \times 10^2$  micromoles/min/mg for the ordinary pyruvate kinase reaction with ADP present. The fact that commercial preparations of rabbit skeletal muscle pyruvate kinase also catalyze the hydrolysis of phosphoenolpyruvate suggests that this effect may account for the previous reports of a lower binding number for phosphoenolpyruvate.

Electrophoresis of various bovine tissue extracts revealed, in addition to the three major homotetrameric isozymes of pyruvate kinase ( $\text{K}_4$ ,  $\text{L}_4$ , and  $\text{M}_4$ ), numerous intermediate bands that behave electrophoretically as hybrid isozymes. Kidney, for example contains both K-L and K-M hybrid sets. Representative hybrids from each set, tentatively identified as  $\text{K}_2\text{L}_2$  and  $\text{K}_3\text{M}$ , were isolated from bovine kidney by ion-exchange chromatography and their subunit compositions were confirmed by dissociation and subsequent

reassociation into new hybrid sets. All of the tissues examined that contain type  $K_4$  also have substantial quantities of K-M hybrids, establishing the presence of the type M isozyme in a great many tissues other than striated muscle and brain, where it is most abundant. In addition, small quantities of K subunits apparently are produced even in striated muscle, which previously had been thought to contain only  $M_4$ . The extensive occurrence of K-M hybrids in bovine tissues indicates that the distribution of mammalian pyruvate kinase isozymes is much more complex than previously reported.

Type  $K_4$  pyruvate kinase was partially purified from bovine kidney, but the presence of substantial quantities of the hybrid isozyme  $K_3M$  in all adult tissues examined precluded the preparation of workable quantities of pure bovine  $K_4$ . Experiments performed on the partially purified preparations revealed the following: the velocity profile as a function of the concentration of the substrate phosphoenolpyruvate is slightly sigmoidal, and the enzyme is strongly inhibited by low levels of L-phenylalanine and L-alanine; and antiserum prepared against bovine type  $M_4$  pyruvate kinase precipitates type  $K_4$ . In these respects, the properties of bovine kidney type  $K_4$  pyruvate kinase closely resemble those reported for type  $K_4$  from other mammalian sources.

Electrophoresis of adult and fetal chicken tissue extracts revealed the presence of only two isozymes instead of the three found in

mammals. These isozymes correspond to the mammalian type K and type M isozymes in their developmental pattern, tissue distribution, electrophoretic, immunological, and kinetic properties. No kinetic, immunological, or electrophoretic evidence could be found for a chicken isozyme corresponding to the mammalian type L pyruvate kinase. In those tissues where type L is found in mammals, type K alone was found in the corresponding chicken tissues. One can speculate that gluconeogenesis in chicken tissues may be adequately supported by type K without the need for a specialized (type L) isozyme as in mammals, and that the properties of chicken type K represent a compromise between the characteristics needed for gluconeogenic and nongluconeogenic tissues.

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## DEDICATION

To my father

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# PYRUVATE KINASE ISOZYMES IN BEEF AND CHICKEN

## INTRODUCTION

### Pyruvate Kinase

The enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, E. C. 2. 7. 1. 40) has been and continues to be the subject of considerable study and interest due to its central role in glycolysis and gluconeogenesis. Pyruvate kinase has been studied extensively, and the work on the enzyme has been reviewed twice (Boyer, 1962; Kayne, 1973). Much of the work has been done on the enzyme from rabbit skeletal muscle, but the pyruvate kinases from other sources have been receiving increasing attention in recent years.

Pyruvate kinase catalyzes the transfer of phosphate from phosphoenolpyruvate (P-enolpyruvate) to ADP. The products are pyruvate and ATP. A monovalent cation ( $K^+$  is the most effective) and a divalent cation (such as  $Mg^{2+}$  or  $Mn^{2+}$ ) are required for activity. Equilibrium for the reaction lies overwhelmingly in the direction of the products ATP and pyruvate, with an equilibrium constant approximately equal to  $6.5 \times 10^3$  at pH 7.4 (McQuate and Utter, 1959).

One of the aspects which attracts considerable interest in pyruvate kinase is its role as a control enzyme during gluconeogenesis. Gluconeogenesis, in the major gluconeogenic tissues, occurs not by the reversal of the pyruvate kinase reaction, which is energetically

very unfavorable, but by a two-step synthesis of P-enolpyruvate from pyruvate, using the intermediate oxaloacetate (Scrutton and Utter, 1968). The two reactions involved, catalyzed by pyruvate carboxylase and P-enolpyruvate carboxykinase, each utilize one high energy phosphate equivalent, making the process energetically favorable. This bypass route around pyruvate kinase requires that the activity of pyruvate kinase be reduced as much as possible in order to avoid the degradation of the newly formed P-enolpyruvate. As will be discussed in greater detail later, the isozyme of pyruvate kinase found in gluconeogenic tissues has control properties that enable its activity to be greatly reduced during conditions of gluconeogenesis.

### Isozymes of Pyruvate Kinase

Although pyruvate kinase, primarily from rabbit skeletal muscle, had been studied extensively for years, the existence of at least three distinct isozymes of pyruvate kinase has been demonstrated only recently. In 1963, von Fellenberg et al. showed on electrophoresis that isozymes differing from the muscle pyruvate kinase exist in rat liver and kidney. Koler et al. (1964) reported a number of molecular differences between pyruvate kinase of leukocytes and erythrocytes in humans. In 1967, Tanaka et al. reported the isolation and characterization of the major isozyme of rat liver

and compared the properties of the liver and muscle isozymes.

Electrophoresis of kidney and liver extracts by Tanaka et al. (1967), however, suggested the presence of skeletal muscle pyruvate kinase as the major form in kidney and the minor form in liver. The apparent presence of the same muscle form of pyruvate kinase in human kidney, liver, leukocytes, striated muscle, and brain was reported by Bigley et al. (1968). However, by including fructose 1,6-diphosphate in the electrophoresis buffer, Susor and Rutter (1968, 1971) were able to demonstrate that this "muscle form" of the enzyme could be resolved into two different isozymes, one found in skeletal muscle, heart, and brain, and the other found in kidney, lung, spleen, testis, and as a minor component of heart, brain, and liver. Thus, three distinct isozymes were identified.

The presence of a total of three isozymes of pyruvate kinase in rat tissues was corroborated by the work of Imamura and Tanaka (1972), who also found a similar pattern in human tissues. In addition, they performed studies using fetal rat tissues, showing that the "muscle form" found in kidney and liver was also probably the sole isozyme of the early fetus. Imamura and Tanaka (1972), therefore, suggested that the fetal form (which they designated type  $M_2$ , but which I will refer to as type K) is a prototype form of pyruvate kinase and that the skeletal muscle form (type  $M_1$ , or simply type M) and the liver form (type L) are differentiated isozymes of pyruvate kinase.



Since this earlier work establishing the existence of at least three isozymes of pyruvate kinase in mammalian tissues, a number of further electrophoretic studies have appeared. The electrophoretic results for pyruvate kinase in rat tissues and tumors obtained by Shapira and Gregori (1971), by Farron et al. (1972), and by Ibsen and Kreuger (1973) indicate three isozymes, and in retrospect the limited electrophoretic data of Pogson (1968a) are also consistent with this view. Comparative studies of the isozyme pattern in rats with those in a number of other mammals have been carried out by Osterman and Fritz (1973) and by Whittell et al. (1973), both concluding that the same patterns exist in a number of mammalian species.

Electrophoretic studies tracing the isozyme patterns during pre- and postnatal life have been done in the rat (Osterman et al., 1973; Walker and Potter, 1972; Walker, 1974) and in the guinea pig (Faulkner and Jones, 1975a). The electrophoretic pattern in tissues of 11-16 week human fetuses was studied by Faulkner and Jones (1975b). Type K pyruvate kinase is the predominant isozyme found in all early fetal tissues examined. The major adult liver isozyme (type L) appears in fetal rat liver shortly before birth and increases greatly after weaning. A shift from type K to the skeletal muscle isozyme (type M) occurs in skeletal muscle, cardiac muscle, and brain as fetal and neonatal development progresses.

As will be discussed in greater detail in a later section,

electrophoresis of various mammalian tissues reveals a number of intermediate bands of lesser intensity in addition to the three major bands of pyruvate kinase. These intermediate bands appear to be hybrids of the three pyruvate kinase isozymes. This thesis will confirm that these bands do correspond to hybrids and that, in fact, hybrid isozymes are present in most bovine tissues. The type K subunit, which predominates in early fetal tissues, probably continues to be synthesized in most or all adult bovine tissues.

The pattern of adult tissue distribution for the three parent isozymes of pyruvate kinase that emerges from the electrophoretic studies in the rat and other mammals is as follows: The type K isozyme, which is the predominant and probably the sole isozyme in the early fetus, continues to be found in most or all adult tissues; a second isozyme (type M) is found in abundance in highly glycolytic tissues, namely skeletal muscle, heart, and brain; and there is a third isozyme (type L) that is found in tissues which are thought to carry on the bulk of gluconeogenesis in mammals, namely liver, kidney, and intestines.

Erythrocyte pyruvate kinase, according to some reports, is the same as type L pyruvate kinase (Bigley et al., 1968; Ibsen et al., 1975). However, other reports state that the electrophoretic mobility of erythrocyte pyruvate kinase is different from type L (Imamura and Tanaka, 1972; Susor and Rutter, 1971; Whittell et al., 1973;

Nakashima, 1974). Nakashima (1974) has found that the electrophoretic mobility of erythrocyte pyruvate kinase may be the same or different from that of type L under various conditions. At present, the nature of erythrocyte pyruvate kinase remains controversial, the suggestions being that it may be a fourth isozyme of pyruvate kinase (Imamura and Tanaka, 1972; Susor and Rutter, 1971), a hybrid (Whittell et al., 1973; Peterson et al., 1974) or merely an electrophoretic variant of type L (Bigley et al., 1968; Ibsen et al., 1975).

A number of different systems have been used to designate the three isozymes of pyruvate kinase. They are compared in Table I. The A-B-C system was first used (Susor and Rutter, 1968). However, this nomenclature does not indicate either relative electrophoretic mobility or tissue distribution. In this thesis the K-L-M system has been adopted, primarily for its mnemonic usefulness (K for kidney, L for liver, and M for muscle, indicating the tissues in which each predominates). A system using letters is also essential for ease in naming hybrid isozymes.

Table I. Principal systems used for designating pyruvate kinase isozymes.

| Relative electrophoretic mobility | Systems |                |   |   |     |
|-----------------------------------|---------|----------------|---|---|-----|
|                                   |         |                |   |   |     |
| Closest to anode                  | L       | L              | B | L | I   |
| Intermediate                      | M       | M <sub>1</sub> | A | M | II  |
| Farthest from anode               | K       | M <sub>2</sub> | C | A | III |

As indicated already, some tissues have more than one isozyme. In the case of liver, type L has been shown to occur only in the parenchymal cells, while type K is only in the non-parenchymal cells (mostly Kupffer cells) (Crisp and Pogson, 1972; Van Berkel et al., 1972).

Fetal isozymes of several enzymes, including pyruvate kinase, are found in tumors (Shapira and Gregori, 1971; Farron et al., 1972; Balinsky et al., 1973a; Farina et al., 1974). Poorly differentiated hepatomas, for example, have mostly or only type K pyruvate kinase instead of the type L of normal liver. The shift in hepatomas from the normal liver pattern to the fetal pattern is less complete in better differentiated lines (Walker and Potter, 1972; Farina et al., 1974).

A shift toward the fetal isozyme pattern is also seen in regenerating liver after partial hepatectomy. As the liver regenerates, the ratio of type K to type L pyruvate kinase increases (Tanaka et al., 1967). This increase in type K actually occurs in the parenchymal cells (Bonney et al., 1973; Garnett et al., 1974); i.e., the cells normally containing only type L pyruvate kinase begin also to produce type K.

Cells in culture also seem to shift to the fetal form of pyruvate kinase, as demonstrated with liver cells in culture (Walker and Potter, 1973).

The idea that there are three distinct isozymes of pyruvate

kinase, as suggested by the electrophoretic patterns, is supported by an examination of the properties of the individual isozymes. The principal distinguishing characteristics are kinetic, but there are also certain physical and immunological differences.

### Properties of Type M Pyruvate Kinase

The skeletal muscle isozyme of pyruvate kinase has been studied most extensively, at first because it was the only one recognized and more recently because of its easy availability and relative stability. The reviews by Boyer (1962) and Kayne (1973) deal largely with the skeletal muscle isozyme. Since the review by Kayne (1973), pyruvate kinases from the skeletal muscle of the beef (Cardenas et al., 1973) and chicken (Cardenas et al., 1975b) have been purified and characterized, and X-ray crystallographic data on the cat muscle pyruvate kinase have been reported (Stammers and Muirhead, 1975).

The molecular weights of skeletal muscle pyruvate kinase range from 212,000 for chicken (Cardenas et al., 1975b) to 250,000 for rat (Tanaka et al., 1967). The rabbit skeletal muscle enzyme has been shown to consist of four subunits of identical or nearly identical molecular weight (Steinmetz and Deal, 1966; Cottam et al., 1969), and this was subsequently shown to be true for the enzyme from frog, beef, and chicken as well (Flanders et al., 1971; Cardenas et al., 1973; Cardenas et al., 1975b). X-ray diffraction data have been obtained on the

skeletal muscle enzyme from rabbit (McPherson and Rich, 1972a,b) and from cat (Stammers and Muirhead, 1975), and the results are consistent with the hypothesis of four identical or nearly identical subunits.

Under normal assay conditions, the skeletal muscle pyruvate kinases have hyperbolic (Michaelis-Menten) kinetics with either substrate, ADP or P-enolpyruvate. Fructose 1,6-diphosphate, which has an effect on the other isozymes, does not affect the kinetics of type M pyruvate kinase under normal conditions. It does, however, have an effect on the inhibition of type M by L-phenylalanine, as will be discussed in a later section.

#### Properties of Type L Pyruvate Kinase

Type L pyruvate kinase has been purified from the livers of rat (Tanaka et al., 1967), beef (Cardenas and Dyson, 1973), and pig (Kutzbach et al., 1973). Tanaka et al. (1967) estimated a molecular weight of 208,000 for rat liver pyruvate kinase, while Kutzbach et al. (1973) reported a molecular weight of 202,000 for the pig liver enzyme. Bovine liver pyruvate kinase has a molecular weight of 215,000 (Cardenas and Dyson, 1973). Both the porcine and bovine liver enzymes have been shown to dissociate into subunits of identical or nearly identical molecular weight. The liver isozyme has sigmoidal kinetics with respect to the substrate P-enolpyruvate, and it is

activated by fructose 1,6-diphosphate to give hyperbolic kinetics with respect to P-enolpyruvate. Hill coefficients for the unactivated enzyme are typically 2.3-2.5, with higher values obtained in the presence of inhibitors such as ATP or L-phenylalanine. The kinetics of type L pyruvate kinase with respect to ADP will be discussed in a later section.

### Properties of Type K Pyruvate Kinase

Type K pyruvate kinase has been purified only by Imamura et al. (1972). It was isolated from Yoshida ascites hepatoma cells of rats and had sigmoidal kinetics with respect to P-enolpyruvate and activation by fructose 1,6-diphosphate. The molecular weight was estimated at 216,000 by gel filtration, placing it intermediate in molecular weight between type M and type L. An extensive investigation of the kinetic properties of this purified type K pyruvate kinase was performed, but further physical studies, such as sedimentation equilibrium, subunit structure, and amino acid analysis, were not done.

Many investigations of the kinetic properties of partially purified preparations of type K pyruvate kinase have been carried out. These are summarized in Table II. Results for the reaction velocity with respect to P-enolpyruvate vary considerably, some workers reporting hyperbolic kinetics, some sigmoidal with activation by fructose 1,6-diphosphate, and still others sigmoidal kinetics without activation by

Table II. Enzymatic velocity with respect to P-enolpyruvate for type K pyruvate kinase from various mammalian sources.

| Tissue                   | Animal | Extent of Purification <sup>a</sup>                                  | pH of Assay | Velocity Profile | Hill coefficient | Fru-1, 6-P <sub>2</sub> Activation | Reference                             |
|--------------------------|--------|--|-------------|------------------|------------------|------------------------------------|---------------------------------------|
| Yoshida ascites hepatoma | Rat    | To purity  | 7.4         | Sigmoidal        | 1.4-1.5          | Yes                                | Imamura <u>et al.</u> , 1972          |
| Ehrlich ascites          | Rat    | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , G-200              | 7.2         | Hyperbolic       | 0.96             | not done                           | Sparmann <u>et al.</u> , 1973         |
| Hepatoma                 | Rat    | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , G-200, P-Cell      | 7.5         | Hyperbolic       |                  | No                                 | Taylor <u>et al.</u> , 1969           |
| Hepatoma                 | Human  | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , acetone cut, G-200 | 7.4         | Hyperbolic       |                  | No                                 | Balinsky <u>et al.</u> , 1973c        |
| Ehrlich ascites tumors   | Mouse  | None   | 7.4         | Interconvertible |                  | not done                           | Feliú <u>et al.</u> , 1975            |
| Kidney cortex            | Rat    | DEAE   | 7.5         | Sigmoidal        | 1.8              | No                                 | Jiménez de Asúa <u>et al.</u> , 1971b |
| Kidney cortex            | Rat    | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , CM                 | 7.5         | Sigmoidal        | 1.7              | No                                 | Costa <u>et al.</u> , 1972            |
| Kidney cortex            | Rat    | Isoelectric focusing   |             | Sigmoidal        | 2-3              | Yes                                | Ibsen and Trippet, 1973               |
| Kidney cortex            | Rabbit | DEAE   | 7.5         | Sigmoidal        | 2.06             | not done                           | Kozhevnikova, 1973                    |



Table II. (Continued)

| Tissue                 | Animal     | Extent of Purification <sup>a</sup>                                  | pH of Assay | Velocity Profile     | Hill coefficient | Fru-1, 6-P <sub>2</sub> Activation | Reference                             |
|------------------------|------------|--|-------------|----------------------|------------------|------------------------------------|---------------------------------------|
| Kidney medulla         | Rabbit     | DEAE   | 7.5         | Hyperbolic           | 1.0              | not done                           | Kozhevnikova, 1973                    |
| Liver or kidney        | Rat        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , dialysis           | 7.0         | Sigmoidal (slightly) |                  | Yes                                | Carbonell <u>et al.</u> , 1973        |
| Liver                  | Human      | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , dialysis           | 7.0         | not done             |                  | Yes                                | Carbonell <u>et al.</u> , 1973        |
| Liver                  | Rat        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , CM                 | 7.5         | Hyperbolic           |                  | No                                 | Jiménez de Asúa <u>et al.</u> , 1971a |
| Liver                  | Rat        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , CM, DEAE           | 7.0         | Hyperbolic           | 1.0              | No                                 | Middleton and Walker, 1972            |
| Liver                  | Rat        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                      | 7.5         | Sigmoidal            | 1.3              | Slight                             | Van Berkel <u>et al.</u> , 1973b      |
| Liver                  | Rat        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                      | 8.0         | Sigmoidal            | 1.7              | Yes                                | Van Berkel <u>et al.</u> , 1973b      |
| Liver                  | Rabbit     | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                      | 7.4         | Hyperbolic           |                  | No                                 | Irving and Williams, 1973             |
| Liver                  | Guinea pig | DEAE   | 7.5         | Complex              | 0.4-2.0          | Yes                                | Faulkner and Jones, 1975a             |
| Fetal liver            | Human      | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , acetone cut, G-200 | 7.4         | Hyperbolic           |                  | No                                 | Balinsky <u>et al.</u> , 1973c        |
| Liver cells in culture | Rat        | None   | 7.5         | Interconvertible     |                  | No                                 | Walker and Potter, 1973               |

Table II. (Continued)

| Tissue                | Animal | Extent of Purification <sup>a</sup>                        | pH of Assay | Velocity Profile     | Hill coefficient | Fru-1, 6-P <sub>2</sub> Activation | Reference                        |
|-----------------------|--------|--|-------------|----------------------|------------------|------------------------------------|----------------------------------|
| Lung cells in culture | Human  | Dialysis   | 7.5         | Hyperbolic           |                  | not done                           | Dunaway and Smith, 1971          |
| Adipose tissue        | Rat    | 40,000 x g supnt.  | 7.4         | Interconvertible     |                  | Partial                            | Pogson, 1968b                    |
| Adipose tissue        | Rat    | None   |             | Hyperbolic           |                  | not done                           | Marco <u>et al.</u> , 1971       |
| Intestinal mucosa     | Rat    | None   | 7.5         | Sigmoidal (biphasic) | 1.2 & 2.1        | Yes                                | Van Berkel <u>et al.</u> , 1974b |
| Leukocytes            | Human  | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ,<br>G-200 |             | Hyperbolic           |                  | not done                           | Koler <u>et al.</u> , 1964       |
| Leukocytes            | Human  | None   | 7.5         | Hyperbolic           | 1.1              | No                                 | Van Berkel and Koster, 1973      |
| Leukocytes            | Human  | None   | 8.5         | Hyperbolic           | 1.2              | No                                 | Van Berkel and Koster, 1973      |

<sup>a</sup> Abbreviations used to indicate the steps used in purification are as follows: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, fractionation with ammonium sulfate; acetone cut, fractionation with acetone; G-200, gel column chromatography with Sephadex G-200; DEAE, ion-exchange chromatography with DEAE-Sephadex or DEAE-cellulose; CM, ion-exchange chromatography with CM-Sephadex or CM-cellulose; P-Cell, ion-exchange chromatography with phosphocellulose.

fructose 1,6-diphosphate. There are also several reports of inter-conversion of type K pyruvate kinase from hyperbolic kinetics with respect to P-enolpyruvate to sigmoidal kinetics, depending on the extraction medium, growth medium for cells in culture, or type of preincubation of the extract.

One explanation has been offered by Van Berkel et al. (1973b) to account for the discrepancies in the reported kinetics of type K pyruvate kinase. Working with the isozyme from rat liver, they found that at pH 7.5 the kinetics were only slightly sigmoidal, whereas at pH 8.0 the kinetics were much more sigmoidal. (Rozenfurt et al. (1969) and Kutzbach et al. (1973) have demonstrated a similar effect of pH on the kinetics of type L pyruvate kinase.) Since most of the kinetic profiles for type K pyruvate kinase from rat liver have been obtained at pH 7.0 or 7.5, it is not surprising that earlier reports have indicated hyperbolic profiles for this isozyme. Unfortunately, this hypothesis does not hold for leukocytes, as Van Berkel and Koster (1973) report hyperbolic velocity profiles both at pH 7.5 and pH 8.5.

There is, of course, the definite possibility that what appears electrophoretically to be the same isozyme (type K) in different tissues may in fact be different isozymes, having different kinetic and other properties. This idea is supported in part by the fact that the kinetics of type K from rat kidney cortex have consistently been reported to be sigmoidal, whereas the kinetics of type K from rat liver are

reported to be hyperbolic or only slightly sigmoidal at pH 7.5 (see Table II). It is impossible to tell from published work whether there is in fact more than one isozyme or hybrid isozyme being labeled type K.

The differences in kinetics of the type K pyruvate kinase studied by various workers may also be due to changes in the enzyme that occur during isolation and purification. The principal purification methods used in each case are summarized in Table II. There does not, however, appear to be any correlation between the purification procedures and the kinetic patterns.

This thesis will show that hybrid isozymes occur to a much greater extent than previously recognized. In many of the published works on type K pyruvate kinase, no electrophoretic studies of the number of isozymes or hybrid isozymes present in the enzyme preparations were undertaken. The possibility that other isozymes or hybrid isozymes having different kinetics were not separated in the purification procedures clouds much of the published results.

#### Interconvertible Forms

Interconvertibility of type K pyruvate kinase between a form having hyperbolic kinetics with P-enolpyruvate and a form having sigmoidal kinetics has been reported in adipose tissue (Pogson, 1968a, b), in Ehrlich ascites tumor cells (Feliú *et al.*, 1975), and

in liver cells in culture (Walker and Potter, 1973). Evidence has also been reported for the existence of interconvertible forms of type K pyruvate kinase in intestinal mucosa (Van Berkel et al., 1974b) and in rat liver (Van Berkel, 1974).

Pogson (1968b) reported that isolation of pyruvate kinase from rat adipose tissue with medium containing EDTA yielded an enzyme which he called PyK-A that has a low affinity for the substrate P-enolpyruvate, sigmoidal kinetics, and activation by fructose 1,6-diphosphate. The activation by fructose 1,6-diphosphate was minimal, however. Extraction in a medium not containing EDTA yielded an enzyme which he called PyK-B that has a high affinity for P-enolpyruvate, hyperbolic kinetics, and insensitivity to fructose 1,6-diphosphate. The conversion of PyK-A into PyK-B could be accomplished by incubation with fructose 1,6-diphosphate. Conversion of PyK-B into PyK-A could be done by incubation with EDTA, ATP, or citrate.

Walker and Potter (1973) extracted pyruvate kinase from rat liver cells in culture and found that the culture conditions of the cells affected the properties of the pyruvate kinase. Cells grown in a medium without glucose yielded an enzyme with a sigmoidal kinetics, whereas cells grown in a medium with glucose gave a pyruvate kinase with hyperbolic kinetics. These two forms corresponded to the PyK-A and PyK-B reported by Pogson. The high affinity form (PyK-B) is

found under cell conditions in which fructose 1, 6-diphosphate is high (glycolytic conditions). Walker and Potter (1973) showed that, as in the case of the adipose tissue pyruvate kinase, the two forms of the pyruvate kinase extracted from cultured liver cells could be inter-converted in vitro by incubation with fructose 1, 6-diphosphate or  $Mg^{2+}$  for the A to B transition or by incubation with EDTA, ATP, or citrate for the B to A transition. The same sort of difference in pyruvate kinase was reported for Ehrlich ascites tumor cells grown with and without glucose (Feliú et al., 1975).

Van Berkel has obtained biphasic kinetic plots in rat intestinal cells (Van Berkel et al., 1974b) and in rat liver type K preparations (Van Berkel, 1974) which he interpreted as indicating an equilibrium mixture of the two interconvertible forms reported by Pogson (1968b). He has investigated the effect of free  $Mg^{2+}$  concentration on the kinetics, with results indicating that the A (inactive) form is introduced by lowering the free  $Mg^{2+}$  concentration.

Pogson (1968b), Walker and Potter (1973), and Van Berkel (1974) state that the interconversion of the PyK-A and PyK-B forms is a relatively slow process compared to the conversion of type L pyruvate kinase from sigmoidal kinetics to hyperbolic kinetics by fructose 1, 6-diphosphate, and that the interconversion may have a physiological function in regulation of pyruvate kinase activity.

The liver type L and erythrocyte pyruvate kinases are also reported to have interconvertible forms. Two different kinetic forms have been obtained with partially purified human erythrocyte pyruvate

kinase depending on the presence or absence of EDTA, similar to the interconvertible forms of type K (Badwey and Westhead, 1975). A different sort of interconvertibility is obtained by altering the redox potential in preparations of rat liver type L and of erythrocyte pyruvate kinase (Van Berkel et al., 1973a, c; Badwey and Westhead, 1975). Van Berkel et al. (1973c) have suggested that abnormal redox potential in erythrocytes may be the cause of pyruvate kinase deficiency, but this viewpoint has been challenged by Blume et al. (1974).

#### Inhibition by Amino Acids

The three isozymes of pyruvate kinase have distinct differences with respect to their inhibition by various amino acids. Type M is the least affected by amino acids, whereas type K is most sensitive, both in degree of inhibition and in the number of amino acids affecting it. Type L pyruvate kinase is also inhibited by many amino acids but to a somewhat lesser extent than is type K.

Rabbit skeletal muscle pyruvate kinase (type M) is inhibited by high levels of L-phenylalanine (Carminatti et al., 1971; Kayne and Price, 1972, 1973). The inhibition is overcome by the presence of L-alanine, L-cysteine, or L-serine. Plots of enzymatic velocity with respect to L-phenylalanine concentration are sigmoidal, although the plots of velocity with respect to P-enolpyruvate retain their normal hyperbolic shape in the presence of L-phenylalanine. The

binding of L-phenylalanine to this enzyme has been shown to be co-operative (Kayne and Price, 1973). It is concluded that L-phenylalanine acts on the enzyme at an allosteric site.

L-Phenylalanine inhibition of rat type M pyruvate kinase has also been demonstrated (Jiménez de Asúa *et al.*, 1971a; Ibsen and Trippet, 1974). The inhibition is reversed by fructose 1,6-diphosphate, L-alanine, L-serine, and other amino acids. L-Alanine alone has no inhibitory effect on the enzyme. Guinea pig muscle pyruvate kinase has similar properties (Faulkner and Jones, 1975).

Bovine type M pyruvate kinase is also very similar to the corresponding rat isozyme, having a  $K_i$  for L-phenylalanine of 22 mM at 0.5 mM P-enolpyruvate and reversal of the inhibition by fructose 1,6-diphosphate or L-alanine (Cardenas *et al.*, 1975c). In addition, velocity plots with respect to P-enolpyruvate are sigmoidal in the presence of L-phenylalanine, having a Hill coefficient of 1.9 when the L-phenylalanine concentration is 5 mM.

In contrast to type M pyruvate kinase, type K pyruvate kinase is strongly inhibited by relatively low concentrations of both L-phenylalanine and L-alanine, as well as moderately inhibited by various other amino acids. A number of the reports on inhibition of type K pyruvate kinase by L-phenylalanine and L-alanine are summarized in Table III. Some workers have investigated the inhibitory effects of other amino acids besides L-phenylalanine and L-alanine, finding



Table III. Inhibition of type K pyruvate kinase by amino acids.

| Tissue                             | Animal | [P-enolpyruvate]<br>(mM) | Amino<br>Acid               | Extent of<br>Inhibition              | Reversal by<br>Fru-1, 6-P <sub>2</sub> | Reference                                |
|------------------------------------|--------|--------------------------|-----------------------------|--------------------------------------|--|--|
| Hepatoma                           | Rat    |                          | Ala                         | $K_i = 0.25$ mM                      | Partially                              | Taylor <i>et al.</i> , 1969              |
| Hepatoma                           | Rat    |                          | Ala<br>Phe                  | $K_i = 0.6$ mM<br>$K_i = 0.5$ mM     | No<br>not done                         | Imamura <i>et al.</i> , 1972             |
| Hepatoma &<br>fetal liver          | Human  | 0.5                      | Ala                         | Strong                               | Yes                                    | Balinsky <i>et al.</i> , 1973c           |
| Kidney<br>cortex                   | Rat    |                          | Ala                         |                                      | No                                     | Jiménez de Asúa <i>et al.</i> ,<br>1971b |
| Kidney<br>cortex                   | Rat    |                          | Ala<br>Phe<br>others        | Strong                               | No                                     | Costa <i>et al.</i> , 1972               |
| Kidney<br>cortex                   | Rat    | 0.34                     | Ala<br>Phe<br>others        | Strong<br>Strong<br>Moderate         | Yes<br>Yes                             | Ibsen and Trippet, 1974                  |
| Kidney, liver, &<br>adipose tissue | Rat    | 0.5                      | Ala<br>Phe<br>Cys<br>others | $K_i = 0.1$ mM<br>Strong<br>Moderate | Yes<br>Yes<br>Yes                      | Carbonell <i>et al.</i> , 1973           |
| Kidney                             | Rabbit | 2.2                      | Phe<br>Ala<br>others        | Strong<br>Strong                     | No<br>No                               | Il'in and Kozhevnikova,<br>1974          |
| Adipose tissue                     | Rat    |                          | Ala                         | Strong                               |  | Marco <i>et al.</i> , 1971               |

Table III. (Continued)

| Tissue                    | Animal     | [P-enolpyruvate]<br>(mM) | Amino<br>Acid                | Extent of<br>Inhibition            | Reversal by<br>Fru-1, 6-P <sub>2</sub> | Reference                                |
|---------------------------|------------|--------------------------|------------------------------|------------------------------------|--|--|
| Liver                     | Rat        | 0.15                     | Ala<br>Phe                   | $K_i = 0.06$ mM<br>$K_i = 0.11$ mM |  | Jiménes de Asúa <u>et al.</u> ,<br>1971a |
| Liver                     | Rat        | various                  | Ala                          | pH dependent                       | Yes                                    | Van Berkel <u>et al.</u> , 1973b         |
| Liver                     | Rabbit     | various                  | Ala                          | Strong                             |  | Irving and Williams,<br>1973             |
| Liver                     | Human      | 0.5                      | Ala<br>Phe                   | Strong<br>Strong                   |  | Carbonell <u>et al.</u> , 1973           |
| Liver or lung             | Guinea pig | 0.5 mM                   | Ala                          | $K_i = 0.8$ mM                     | Yes                                    | Faulkner and Jones,<br>1975a             |
| Liver cells<br>in culture | Rat        |                          | Interconvertible Form PyK-A: |                                    |  | Walker and Potter, 1973                  |
|                           |            |                          | Ala                          | $K_i = 0.01$ mM                    | No                                     |  |
|                           |            |                          | Phe                          | $K_i = 0.017$ mM                   | No                                     |  |
|                           |            |                          | Interconvertible Form PyK-B: |                                    |  |  |
|                           |            |                          | Ala                          | $K_i = 0.1$ mM                     | Yes                                    |  |
|                           |            |                          | Phe                          | $K_i = 0.07$ mM                    | Yes                                    |  |
| Leukocytes                | Human      | 0.5<br>0.1               | Ala<br>Phe<br>others         | $K_i = 1.5$ mM<br>$K_i = 1.5$ mM   | Yes<br>Yes                             | Van Berkel and Koster,<br>1973           |

moderate inhibition by L-cysteine, L-proline, L-valine, L-tryptophan, L-isoleucine, L-threonine, L-histidine, and L-leucine (Jiménez de Asúa et al., 1971a; Costa et al., 1972; Carbonell et al., 1973; Ibsen and Trippet, 1974; Il'in and Kozhevnikova, 1974). L-Serine activates type K pyruvate kinase and overcomes inhibition by other amino acids (Ibsen and Trippet, 1974; Il'in and Kozhevnikova, 1974). Some investigators find that fructose 1,6-diphosphate can reverse the inhibition of type K pyruvate kinase by L-phenylalanine and L-alanine, but others state that this does not occur. This inconsistency may perhaps be explained by the existence of interconvertible forms. Evidence that L-alanine causes a dissociation of type K pyruvate kinase into two half molecules, an effect that is reversed by fructose 1,6-diphosphate, has been presented by Sparmann et al. (1973) and by Schulz et al. (1975).

Rat type L pyruvate kinase is also inhibited by fairly low levels of L-phenylalanine and L-alanine (see Table IV). While the inhibition of type L by L-alanine is approximately the same as that of type K, the type L isozyme is inhibited more than type K by L-cysteine but less than type K by L-phenylalanine (Costa et al., 1972; Carbonell et al., 1973; Ibsen and Trippet, 1974). A number of other amino acids also inhibit the rat type L isozyme (Costa et al., 1972; Ibsen and Trippet, 1974).

In contrast to the type L pyruvate kinase from rat, the same

Table IV. Inhibition of type L pyruvate kinase by amino acids.

| Tissue | Animal     | [P-enolpyruvate]<br>(mM) | Amino<br>Acid                          | Extent of<br>Inhibition                | Reversal by<br>Fru-1, 6-P <sub>2</sub> | Reference                      |
|--------|------------|--------------------------|--|--|--|--------------------------------|
| Liver  | Rat        |                          | Ala                                    | $K_i = 2.0$ mM                         | Yes                                    | Taylor <i>et al.</i> , 1969    |
| Liver  | Rat        |                          | Ala<br>Phe                             | $K_i = 1.0$ mM<br>$K_i = 5$ mM         | Yes<br>not done                        | Imamura <i>et al.</i> , 1972   |
| Liver  | Rat        | 0.5                      | Ala<br>Cys<br>Phe<br>Ala+Phe<br>others | Strong<br>Strong<br>Moderate<br>Strong | Yes                                    | Carbonell <i>et al.</i> , 1973 |
| Liver  | Rat        | 0.34                     | Ala<br>Phe<br>others                   | Strong<br>Moderate                     | Yes<br>Yes                             | Ibsen and Trippet,<br>1974     |
| Liver  | Human      | 0.5                      | Ala<br>Cys                             | Strong<br>Strong                       |  | Carbonell <i>et al.</i> , 1973 |
| Liver  | Human      | 0.5                      | Ala                                    | Strong                                 | Yes                                    | Balinsky <i>et al.</i> , 1973c |
| Liver  | Rabbit     | 0.15                     | Ala                                    | $K_i = 2.5$ mM                         | Yes                                    | Irving and Williams,<br>1973   |
| Liver  | Guinea pig | 0.5                      | Ala<br>Phe                             | Moderate<br>Strong                     | Yes<br>Partial                         | Faulkner and Jones,<br>1975a   |

Table IV. (Continued)

| Tissue           | Animal | [P-enolpyruvate]<br>(mM) | Amino<br>Acid            | Extent of<br>Inhibition              | Reversal by<br>Fru-1, 6-P <sub>2</sub> | Reference                                |
|------------------|--------|--------------------------|--------------------------|--------------------------------------|--|--|
| Liver            | Beef   | 0.5                      | Ala<br>Phe               | Not at all<br>$K_i = 0.49$ mM        | Yes                                    | Cardenas <u>et al.</u> , 1975c           |
| Kidney<br>cortex | Rat    |                          | Ala                      | Strong                               | Yes                                    | Jiménez de Asúa <u>et al.</u> ,<br>1971b |
| Kidney<br>cortex | Rat    |                          | Ala<br>Phe<br>others     | Strong<br>Moderate                   | Yes<br>Yes                             | Costa <u>et al.</u> , 1972               |
| Erythrocytes     | Human  | 0.3                      | Ala<br>Phe<br>Val<br>Pro | Strong<br>Strong<br>Strong<br>Strong |  | Black and Henderson,<br>1972             |

isozyme from guinea pig shows only moderate inhibition by L-alanine (Faulkner and Jones, 1975), and the type L isozyme from beef is only slightly inhibited by L-alanine (Cardenas et al., 1975c).

Several papers have compared the inhibitions of all three isozymes by amino acids (Taylor et al., 1969; Imamura et al., 1972; Carbonell et al., 1973; Ibsen and Trippet, 1974; Faulkner and Jones, 1975).

For purposes of comparing the inhibitions of different isozymes by a given amino acid, or comparing the same isozyme in different species, Cardenas et al. (1975c) have pointed out the necessity of using the same concentration of P-enolpyruvate, since the degree of inhibition at a given concentration of amino acid varies considerably depending on the concentration of this substrate.

#### Inhibition by ATP

ATP inhibits all three isozymes. Reynard et al. (1961) found that ATP inhibition of the rabbit skeletal muscle isozyme is overcome competitively by either ADP or P-enolpyruvate. In the rat, type L pyruvate kinase is inhibited by much lower concentrations of ATP than are type K and type M (Imamura et al., 1972; Costa et al., 1972; Ibsen and Trippet, 1973; Carbonell et al., 1973). Typical inhibition constants ( $K_i$ ) for ATP are 0.15 mM for type L, 1.8 mM for type K, and 3.3 mM for type M (Ibsen and Trippet, 1973). The strong

inhibition of type L pyruvate kinase is probably allosteric (Llorente et al., 1970; Costa et al., 1972; Carbonell et al., 1973). Fructose 1,6-diphosphate reverses ATP inhibition of type L and has a partial reversing effect on ATP inhibition of type K (Imamura et al., 1972; Van Berkel et al., 1973b; Carbonell et al., 1973).

Several investigators report that addition of extra  $Mg^{2+}$  reverses ATP inhibition of rat type M, partially reverses that of type K, but does not reverse ATP inhibition of type L (Imamura et al., 1972; Ibsen and Trippet, 1973). They conclude that ATP inhibition of type M, and to a large extent that of type K, is due primarily to binding of  $Mg^{2+}$  by the ATP. However, Boyer (1969), using a  $Mg^{2+}$  "buffer," has established that ATP inhibition of rabbit skeletal muscle pyruvate kinase truly occurs and is not due to removal of free  $Mg^{2+}$  by the ATP. The inhibition of type K by ATP at excess  $Mg^{2+}$  is slight and thought not to be of physiological significance (Van Berkel, 1974).

Among mammals other than the rat, the inhibition of the isozymes by ATP follows a pattern that is for the most part similar. Bovine type M pyruvate kinase is only slightly inhibited by ATP, while bovine type L is very strongly inhibited by ATP (Cardenas et al., 1975a). The isozymes of pyruvate kinase in guinea pig are also very similar to those of the rat with respect to ATP inhibition (Faulkner and Jones, 1975a). The ATP inhibition of human type L (from normal

liver) and of human type K (from hepatoma or from fetal liver) is also very similar to the ATP inhibition of the corresponding rat isozymes (Balinsky et al., 1973c).

#### Inhibition by ADP

The activity of type L pyruvate kinase is inhibited by high concentrations of the substrate ADP when the other substrate, P-enolpyruvate, is at low levels. At P-enolpyruvate concentrations of around 0.2 mM, maximal activity of type L is reached between 0.5 and 1.0 mM ADP. Higher concentrations of ADP inhibit the isozyme. In fact, ADP concentrations of around 5 mM result in more than 90% inhibition of the maximal activity (Tanaka et al., 1967; Kutzbach et al., 1973; Ibsen and Trippet, 1973; Cardenas et al., 1975a). The degree of inhibition by ADP is less at higher concentrations of P-enolpyruvate or when fructose 1,6-diphosphate is present (Kutzbach et al., 1973; Balinsky et al., 1973b; Ibsen and Trippet, 1973). The data of Ibsen and Trippet (1973) indicate that type K pyruvate kinase from rat kidney cortex is slightly inhibited by high concentration of ADP, but this inhibition is less than that seen with the type L isozyme. It should be noted that Irving and Williams (1973) show no inhibition of type L pyruvate kinase in a plot of velocity versus concentration of  $\text{MgADP}^-$  up to concentrations of 8 mM  $\text{MgADP}^-$ . Perhaps this result indicates a complexity about the ADP inhibition that is not yet fully understood.



### Other Activators and Inhibitors

A number of hexose phosphates, in addition to fructose 1,6-diphosphate, and a number of triose phosphates activate the liver type L pyruvate kinase (Van Berkel et al., 1974a; Balinsky et al., 1973c). These activators include fructose 1-phosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, 2-deoxyglucose 6-phosphate, glyceraldehyde 3-phosphate, glycerate 2-phosphate and dihydroxyacetone phosphate. Found not to be effective are galactose 6-phosphate, glycerate 3-phosphate, glycerol 1-phosphate, and glucose and fructose themselves. Some of these same phosphorylated monosaccharides are also reported to activate the pyruvate kinase of erythrocytes (Black and Henderson, 1972). A number of the phosphorylated hexoses were tested with the type K pyruvate kinases of human hepatoma and human fetal liver, but no activation was observed (Balinsky et al., 1973c).

AMP and citrate have an inhibitory effect on human type L and type K pyruvate kinase (Balinsky et al., 1973c). Inhibition by AMP is weaker than the inhibition by citrate or ATP. While AMP inhibits type K approximately the same as type L, citrate inhibits type L more than type K.

### Immunological Relationships among the Isozymes

Imamura et al. (1972) summarized the immunological cross-reactivity of their purified preparations of the three isozymes of rat pyruvate kinase. Type L pyruvate kinase does not cross-react with either of the other two isozymes. In contrast, antibodies to either type M or type K pyruvate kinase will inactivate the other isozyme of this pair. Antiserum to type K pyruvate kinase completely neutralizes the type K isozyme but leaves about 15% of the activity of type M. However, both type K and type M are completely neutralized by M antiserum. These results imply that types K and M pyruvate kinase are immunologically very similar but not identical, while type L is immunologically distinct from types K and M.

### Dietary Regulation of Pyruvate Kinase Isozymes

Tanaka et al. (1967) found that the level of type L pyruvate kinase in rat liver varied according to the nutritional status or dietary intake of the animal. In periods of starvation or on a diet low in carbohydrates, type L pyruvate kinase decreased, whereas on a diet high in carbohydrates the level of this isozyme in the liver increased. The level of the minor liver isozyme (type K) did not fluctuate.

Sandoval and Carbonell (1974) extended this study to rat kidney and reticulocytes. They found dietary regulation of type L pyruvate

kinase but not type K in kidney. Reticulocytes, which still synthesize some proteins, have a level of pyruvate kinase that varies with diet; but erythrocytes, which no longer synthesize protein, were observed to undergo no changes in pyruvate kinase level with diet. These results suggest that type L pyruvate kinase may be subject to dietary regulation in whatever tissue it is found as long as protein synthesis is still possible, but that type K pyruvate kinase is not. However, Osterman and Fritz (1974) reported that all of the isozymes and hybrid isozymes in rat intestines (types K and L plus hybrids having both K and L subunits) appeared to vary in level with dietary changes.

The regulation of type L pyruvate kinase by diet is understandable in view of the presence of type L in tissues in which substantial gluconeogenesis occurs. During periods of gluconeogenesis, pyruvate kinase activity must be reduced in order to diminish the degradation of P-enolpyruvate, newly synthesized by the bypass route through oxaloacetate. When the organism is consuming a low carbohydrate diet, glycolysis is used less and gluconeogenesis occurs much more. A lowering of the total level of type L pyruvate kinase makes the shut-down of pyruvate kinase during such conditions much easier.

#### Hybrid Isozymes

As mentioned earlier, skeletal muscle pyruvate kinase (type M) and the major liver isozyme (type L) are tetramers composed of very

similar if not identical subunits. Hybrid isozymes containing more than one kind of subunit in a tetramer are therefore possible.

Cardenas and Dyson (1973) have formed hybrids in vitro from bovine type M and type L pyruvate kinases. A set of five isozymes was obtained, which corresponded electrophoretically to the five species  $L_4$ ,  $L_3M$ ,  $L_2M_2$ ,  $LM_3$ , and  $M_4$ .  $L_2M_2$  was separated from the others and renatured to yield a five-membered set like the first in order to substantiate that the species obtained were hybrids. Cardenas et al. (1975b) have also reported the in vitro hybridization of chicken type M pyruvate kinase and bovine type L. The hybridizations have resulted in apparently random reassortment of subunits in a binomial distribution, indicating that the type L and type M isozymes are sufficiently similar that selection of like subunits is not appreciably favored.

A naturally occurring set of pyruvate kinase hybrids has been reported in frog eggs (Schloen et al., 1974). Several of the isozymes were rehybridized to generate new sets, confirming that they are indeed naturally occurring hybrids.

Electrophoretic patterns that appear to be K-L hybrid sets have been reported in adult rat kidney, intestines, and sometimes liver (Imamura and Tanaka, 1972), and also in kidneys of mouse, rabbit, guinea pig, and sheep (Whittell et al., 1973). While Osterman et al. (1973) saw only two intermediate bands between  $K_4$  and  $L_4$  in rat kidney instead of the expected three bands, they have published an

extensive report on a full five-membered set from the mucosa of rat small intestine (Osterman and Fritz, 1974). Susor and Rutter (1968) found a K-L hybrid set in fetal rat liver. Recently, however, Walker and Potter (1972) and Walker (1974) have presented evidence for the presence of type M pyruvate kinase in fetal and newborn rat liver possibly associated with the hemopoietic cells. This isozyme is said to disappear from the liver of the newborn rat several days after birth. The type M pyruvate kinase, since it migrates electrophoretically between type K and type L, could be confused with one of the K-L hybrid bands.

The only report of electrophoretic bands that appear to be K-M hybrids in adult mammalian tissues is that of Whittell et al. (1973), who saw two bands suggestive of partial K-M hybrid sets in spleen and testis. In fetal rat brain, a five-membered K-M hybrid set was detected by Susor and Rutter (1968), and up to five bands were also seen in the K-M region on electrophoresis of developing brain and muscle of fetal guinea pig (Faulkner and Jones, 1975a).

The fact that K-L and K-M hybrids seem to exist implies that type K pyruvate kinase, like type M and type L, is composed of four identical or nearly identical subunits.

Since the isozymes are composed of four subunits, and because one wishes to talk about hybrid isozymes, the parental forms of pyruvate kinase can more accurately be referred to as  $K_4$ ,  $L_4$ , and

$M_4$  pyruvate kinase. In this thesis the terms type K and  $K_4$ , type L and  $L_4$ , and type M and  $M_4$  will be used interchangeably to refer to the homotetrameric isozymes.

### Physiological Significance of the Isozymes

Imamura and Tanaka (1972) have suggested that type K pyruvate kinase, because it is probably the sole form of the enzyme found in the early fetus and is found as well in many adult tissues, is the prototype of the pyruvate kinase isozymes. They suggested that we should think of type L and M as differentiated forms, since they increase during differentiation of tissues.

It is true that the properties of type M and type L pyruvate kinase seem to be consistent with the particular needs of the tissues in which they are found. Type M pyruvate kinase is associated with adult tissues that rely heavily on glycolysis for energy production--namely, skeletal muscle, cardiac muscle, and brain. Type M is suited for its role in these tissues, since it has hyperbolic kinetics with a high affinity for P-enolpyruvate and is not significantly inhibited by other metabolites.

Type L pyruvate kinase, on the other hand, is found in tissues responsible for the bulk of gluconeogenesis carried on in an organism--liver, kidney, and possibly jejunal mucosa (Anderson and Rosendall, 1973). Gluconeogenesis in these tissues does not occur by reversal

of the pyruvate kinase reaction but rather by a two-step synthesis of P-enolpyruvate from pyruvate by way of the intermediate oxaloacetate (Scrutton and Utter, 1968). It is necessary that pyruvate kinase activity be shut off or at least substantially reduced in order to avoid the wasteful breakdown of newly synthesized P-enolpyruvate. Without the elimination of P-enolpyruvate breakdown during gluconeogenesis, energy would be needlessly expended, since the synthesis of P-enolpyruvate by the two enzymes--pyruvate carboxylase and P-enolpyruvate carboxykinase--requires two high energy phosphate equivalents while the pyruvate kinase reaction yields only one. Type L pyruvate kinase, in contrast to type M, is definitely a regulatable enzyme and seems to have the control properties which enable its activity to be substantially reduced during gluconeogenesis (Weber, 1969). It is subject to feed-forward activation by the glycolytic metabolite fructose 1,6-diphosphate as well as by other phosphorylated hexoses and trioses. Allosteric inhibition of type L occurs in the presence of the product ATP and in the presence of alanine, which is the transamination product of pyruvate. Citrate and AMP are also inhibitors. Nutritional regulation of the levels of type L pyruvate kinase constitutes an additional regulatory mechanism, as pointed out above. It has been concluded that, at physiological concentrations of substrates and known modifiers, the activity of liver pyruvate kinase (type L) is nearly zero except when activated by a

rise in the concentration of fructose 1,6-diphosphate (Llorente et al., 1970; Flory et al., 1974; Van Berkel et al., 1974a).

Type K pyruvate kinase seems to exhibit many of the same regulatory properties as type L but, for the most part, to a somewhat lesser extent. It has, at least under some conditions, homotropic cooperativity with the substrate P-enolpyruvate, inhibition by amino acids, citrate, and AMP, and activation by fructose 1,6-diphosphate. Unlike type L, it does not seem to be strongly inhibited by ATP, nor is it activated by the many other phosphorylated hexoses and trioses that affect the type L.

No one seems to have a plausible explanation for the existence of a regulatable isozyme in non-gluconeogenic tissues. Carbonell et al. (1973) have speculated that a regulatable pyruvate kinase may have some use in selected tissues and that the isozyme appears in other tissues fortuitously. Since type K is the prototype isozyme, perhaps it ought to have properties that on the whole are intermediate between those of the differentiated types M and L. However, one can't help wondering why the prototype form of pyruvate kinase is not either type M or type L instead of a third isozyme. Perhaps a clearer understanding of the role of type K pyruvate kinase will be gained by further elucidation of the properties of type K and by further knowledge of the intracellular conditions and metabolic processes of those cells in which it is found.



### The Number of Active Sites on Pyruvate Kinase

The number of active sites on mammalian pyruvate kinase has been the subject of several investigations but with incomplete agreement. Reynard et al. (1961) obtained a value slightly greater than two for the number of P-enolpyruvate molecules bound per tetramer of rabbit skeletal muscle pyruvate kinase, using the techniques of equilibrium dialysis and ultracentrifugation. They also found that the number of pyruvate molecules bound was between two and four. A value of two  $\text{Mn}^{2+}$  bound per tetramer of pyruvate kinase was reported by Mildvan and Cohn (1965). Steinmetz and Deal (1966) discussed the possibility that pyruvate kinase could be a dimer of dimers, based on dissociation of the tetramer into active dimers, and interpreted the results as supporting the existence of two active sites. Additional support for this scheme came from the report of Betts and Evans (1968) that about two P-enolpyruvate and two ADP are bound per molecule, using gel filtration.

However, in 1971, Cottam and Mildvan revised the estimation of the number of  $\text{Mn}^{2+}$  binding sites to four, using freshly prepared enzyme. They found that prolonged storage of the enzyme resulted in a drop in specific activity from 280 to 100.

Also consistent with the idea of four active sites on pyruvate kinase were the reports of Kayne (1971) and of Hollenberg et al. (1971). Kayne (1971) carried out an equilibrium dialysis study of

the binding of radioactive thallium-204, making use of the fact that thallium substitutes for  $K^+$  in activating pyruvate kinase, and obtained a Scatchard plot giving a value of four  $Tl^+$  binding sites. He also carried out equilibrium dialysis experiments with radioactive P-enolpyruvate at saturating levels of P-enolpyruvate (up to 0.5 mM), obtaining an average of 3.8 molecules bound per enzyme. Hollenberg *et al.* (1971) found four reactive lysyl  $\epsilon$ -amino groups to be implicated in ADP binding.

Thus, there has been some disagreement as to the probable number of active sites on pyruvate kinase. As Kayne (1971) has pointed out, the simplest scheme would be that pyruvate kinase has four binding sites for all substrates, cofactors, and effector molecules--*i. e.*, one of each type of binding site per subunit. Much of the data are consistent with this view, but in order to provide additional experimental data, part of the work of this thesis has been devoted to performing equilibrium binding studies of P-enolpyruvate to bovine skeletal muscle pyruvate kinase.

#### Scope of this Work

The work described in this thesis has been directed to several different areas of investigation, designed to elucidate the properties of the pyruvate kinase isozymes. First, in an effort to lend support to the idea that there are four active sites per enzyme molecule,

equilibrium binding studies of the substrate P-enolpyruvate to bovine skeletal muscle pyruvate kinase have been undertaken. Four binding sites for P-enolpyruvate per tetramer were found. Second, electrophoretic studies of pyruvate kinase in bovine tissues were undertaken, clearly showing three isozymes of pyruvate kinase in bovine tissues, with a tissue distribution quite similar to that found in the rat. An additional finding from this study has been the demonstration of significant quantities of K-M hybrids in many bovine tissues to an extent not indicated in previous studies of rats or other mammals.

At the same time that electrophoretic studies were being performed on the bovine pyruvate kinase isozymes, an investigation of the electrophoretic patterns of pyruvate kinase isozymes in chicken tissues was undertaken. The electrophoretic results indicate that chicken tissues contain only two isozymes of pyruvate kinase, corresponding to the mammalian types K and M. An isozyme corresponding to the mammalian type L pyruvate kinase does not seem to occur in chickens. Kinetic and immunological studies have corroborated these electrophoretic results.

Finally, attempts to isolate and purify type  $K_4$  pyruvate kinase from bovine kidney are reported. The primary difficulty in obtaining large quantities of pure  $K_4$  is the separation of  $K_4$  from the  $K_3M$  hybrid which is present in significant amounts in all adult bovine tissues containing  $K_4$ . This difficulty has not been overcome, but

some partially purified  $K_4$  has been separated from  $K_3M$  and has been used in kinetic studies.

Much of the work described in this thesis has already been published with permission of the Graduate School of Oregon State University. The equilibrium binding studies with bovine skeletal muscle pyruvate kinase were included in the report by Cardenas et al. (1973). The bovine isozyme patterns were reported in abstract form (Strandholm et al. , 1975a) and formed part of the article by Strandholm et al. 1976). The work on chicken isozymes constituted the article by Strandholm et al. (1975b). Preliminary reports on the latter two subjects were included in the symposium article by Cardenas et al. (1975a). The kinetics of bovine type  $K_4$  pyruvate kinase and its inhibition by L-alanine and L-phenylalanine were part of the article by Cardenas et al. (1975c).

## MATERIALS AND METHODS

### Materials

Biochemical reagents, such as substrates, buffers, serum albumin, and lactate dehydrogenase, were obtained from the Sigma Chemical Co. Sigma was also the source for the Sephadex chromatography gels (CM-Sephadex C-50, DEAE-Sephadex A-50, and Sephadex G-200). Whatman DEAE-Cellulose DE-52 was obtained from Reeve-Angel. The polyacrylamide gels used for the equilibrium binding studies (Bio-Gel P-6 and Bio-Gel P-10) and the dimethyldichlorosilane used for coating the binding column were purchased from Bio-Red. Schwarz-Mann supplied "enzyme-grade" sucrose and ammonium sulfate. All other chemicals were standard reagent grade.

Distilled, deionized water was used for making all solutions. KOH rather than NaOH was also used to raise the pH of a solution, in order to avoid the addition of  $\text{Na}^+$ , which has an inhibitory effect on pyruvate kinase activity.

Cellulose acetate strips (Sephacel III, 1 x 6 in.) for electrophoresis were obtained from the Gelman Instrument Co.

Dialysis tubing, purchased from VWR United, was prepared for use by gentle boiling for 1 to 4 hours in 2 to 4 changes of a solution of approximately 1 mM EDTA and 10 mM  $\text{KHCO}_3$ , followed by a final boil in distilled, deionized water only. The treated dialysis

tubing was rinsed and stored at 4° under water with a trace of chloroform to inhibit growth of microorganisms.

### Animal Tissues

Bovine tissues were obtained from slaughter houses in Corvallis, Philomath, or Portland, Oregon, and placed in ice as soon as possible after death of the animal. The tissues were used the same day or frozen at -80°C until needed. Bovine blood was collected in heparinized bottles. (Approximately 75 units heparin per ml of blood was used. The heparin was dissolved in a minimal quantity of water beforehand.)

Adult male white leghorn chickens were obtained from the Poultry Science Department, Oregon State University. Fertilized white leghorn chicken eggs were purchased from Jenks' Hatchery, Tangent, Oregon, and incubated at 38°C. Adult chickens were bled by heart puncture, and the blood was collected in heparinized tubes. The chicken was then anesthetized with ether, sacrificed, and other tissues were rapidly excised from the chicken and placed on ice. In the adult, femorotibialis muscle was taken from the thigh and pectoralis major muscle from the breast. The entire muscle mass was dissected from breast or thigh of embryos.

For the electrophoresis of mouse pyruvate kinase, C57BC/6 mice (16-day pregnant females) were obtained from the laboratory

of Dr. R. W. Newburgh, Oregon State University.

### Purified Skeletal Muscle Pyruvate Kinase

Pyruvate kinase for use in the equilibrium binding studies was purified from bovine skeletal muscle by the method of Cardenas et al. (1973). Specific activities were usually in the range 200-230 units per mg at 25°. The enzyme was stable for at least six months at 4° as the precipitate in 80% saturated ammonium sulfate containing 0.01 M  $\beta$ -mercaptoethanol.

Pyruvate kinase used in the equilibrium binding studies on gel columns was saved for re-use by dialyzing the fractions containing the enzyme against 80% saturated ammonium sulfate with 0.01 M  $\beta$ -mercaptoethanol and storing at 4°.

Rabbit skeletal muscle pyruvate kinase was obtained from Worthington Biochemical Co. It was found to have a specific activity (micromoles/min/mg) of 170 at 25°.

### Extinction Coefficient of Bovine Skeletal Muscle Pyruvate Kinase

The extinction coefficient of bovine skeletal muscle pyruvate kinase was determined by measuring the absorbance of a solution of the enzyme and then evaporating the solution to dryness at 100° and weighing the protein. A solution containing several milligrams

of the enzyme was exhaustively dialyzed against 0.01 M ammonium acetate, pH 7.0. The absorbance of the solution was read at 280 and 260 nm. Also a synthetic boundary experiment was performed in the analytical ultracentrifuge in order to determine the number of interference fringes produced in the Rayleigh optical system by a given concentration of this enzyme. A carefully measured quantity of the enzyme solution was dried at 85° in a tared vial, which was then placed in a drying pistol containing  $P_2O_5$ . The pistol was evacuated and held at 100° with a steam bath. After several days, the apparatus was cooled to the same temperature as the air in the room containing the balance. Air was admitted to the pistol through an anhydrous  $CaCl_2$  drying tube, and the vial was quickly removed and weighed on the balance, which had an accuracy of  $\pm 10$  micrograms. (The air in the balance case was dried with silica gel beforehand.) The vial was then replaced in the drying pistol, which was evacuated and heated for another day. Repeated measurements were made in this way until successive weighings produced the same value. A parallel experiment was carried out using dialysate only to correct for non-volatile components in the ammonium acetate, the quantity of which was found to be negligible.



### Assays of Pyruvate Kinase Activity

The activity of pyruvate kinase was determined by coupling the pyruvate kinase reaction to that of lactate dehydrogenase according to the method of Bücher and Pfeleiderer (1955). The assay medium consisted of 0.05 M imidazole-HCl (pH 7.0), 0.10 M KCl, 0.01 mM  $\text{MgCl}_2$ , 0.16 mM NADH, 2.0 mM ADP, 1.0 mM P-enolpyruvate, and approximately four units (micromoles of substrate consumed per min) of lactate dehydrogenase per ml. The concentration of P-enolpyruvate in this assay medium was increased from 1.0 mM to 6.0 mM for assays of purified bovine kidney pyruvate kinase isozymes. The total volume of each assay was either 1.0 ml or 0.5 ml. The decrease in absorbance of the assay medium at 340 nm was measured in cuvettes with 4 mm sample width and 10 mm pathlength in a Beckman Acta III recording spectrophotometer equipped with a circulating water bath to maintain the temperature at 25°C.

One unit of pyruvate kinase catalyzes the production of one micromole of pyruvate per min. It is assumed that the lactate dehydrogenase (which is present in excess) converts the pyruvate rapidly and essentially completely to lactate as long as NADH is present. Since the extinction coefficient for NADH is  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ , for our 1.0 cm pathlength cuvettes and a total assay volume of 1.0 ml, the activity of pyruvate kinase may be calculated as

follows:

$$\text{Activity (units)} = \frac{(\Delta A)}{(6.22)(\text{ml enzyme})}$$

where  $\Delta A$  = initial rate of change of absorbance at 340 nm in  
units of absorbance per min

and ml enzyme = the aliquot of enzyme solution added to the  
cuvette (measured in ml).

For 0.5 ml assay volumes, the actual activity would be one-half that  
calculated by the above formula.

Specific activity was expressed as units of pyruvate kinase  
activity per mg of protein.

#### Determination of Protein Concentration

Protein concentrations in unpurified preparations of pyruvate  
kinase were determined with a modified Folin-Ciocalteu method  
(Clark, 1964). Bovine serum albumin was used as a standard. The  
method was adapted for use in the narrow (4.0 mm width and 10 mm  
pathlength) cuvettes by scaling down the volume. The solutions  
and methods were as follows:

Reagent A: 1%  $\text{CuSO}_4$

Reagent B: 2% sodium tartrate

Reagent C: 2%  $\text{Na}_2\text{CO}_3$  in 0.1 M NaOH

Reagents A and C were stored at room temperature; Reagent B at 4°.

On the day of use, 1 ml of reagent A was mixed with 1 ml of reagent B. To this mixture was added 100 ml of reagent C.

Appropriate quantities of samples to be tested were pipetted into test tubes and distilled water was added to each to a total volume of 0.60 ml.. Then to each test tube was added 3.0 ml of the mixture of reagents A, B, and C. After mixing and waiting 10 min or longer, 0.15 ml of 2 N Folin-Ciocalteu phenol reagent (Fischer Scientific Co.) was added to a test tube followed immediately by rapid mixing on a vortex mixer. After 30 min the color could be read in a spectrophotometer. The color is stable up to 24 hr. Usually the color was read at 500 nm, although in certain cases of low protein concentrations in partially purified preparations of pyruvate kinase, the color was read at the more sensitive 700 nm. The standard curve is usually not quite a straight line; however, a typical result gives a line approximated by the following formula:

$$\text{Protein (mg/ml)} = \frac{(A_{500})(0.35)}{(\mu\text{l of sample})}$$

The concentration of purified pyruvate kinase was determined by measuring the absorbance at 280 nm. The extinction coefficient ( $E_{280}^{0.1\%}$ ) of rabbit skeletal muscle pyruvate kinase is 0.54 (Beisenherz et al., 1953). The extinction coefficient ( $E_{280}^{0.1\%}$ ) for bovine skeletal

muscle pyruvate kinase has been determined in this work to be 0.55 (see Results section)--i.e., a 1 mg/ml solution has an absorbance of 0.55.

#### Determination of Concentrations of P-enolpyruvate and ADP

There are two reasons for measuring the concentrations of P-enolpyruvate and ADP: (1) for determining the exact concentrations of the stock solutions used in making the various concentrations of substrates during kinetic studies, and (2) for measuring P-enolpyruvate concentrations in the equilibrium binding studies.

An accurate way of determining the concentrations of these two substrates of pyruvate kinase is to use the coupled enzyme assay system described in a previous section. If a small amount of one of the substrates is added to the assay in the presence of large amounts of pyruvate kinase and the other substrate, the absorbance of the solution will rapidly decline until the small amount of the limiting substrate is used up. The decrease in absorbance of the NADH at 340 nm will be proportional to the quantity of the limiting substrate added to the cuvette.

A problem can arise with this method when measuring P-enolpyruvate solutions which may also contain pyruvate. The added pyruvate will react in the lactate dehydrogenase reaction, causing a decrease in NADH that would be attributed falsely to P-enolpyruvate.

Hence, the sequence of additions to the assay cuvette is important in order to separate the contribution of pyruvate from that of P-enolpyruvate. The methods are summarized in Table V. The concentration in the sample may be calculated with the following formula:

$$\text{Concentration (mM)} = \frac{\Delta A \times \text{final volume (ml)}}{6.22 \times \text{aliquot assayed (ml)}}$$

where  $\Delta A$  is the change in absorbance (after correction for dilution by volume changes, if necessary).

The calculations are more convenient if the final volume after all additions is 1.00 ml.

Method A is the most straightforward method but has two drawbacks. It measures both P-enolpyruvate and pyruvate together and should not be used if pyruvate is present with P-enolpyruvate. Also the volume changes are usually substantial and the initial absorbance must therefore be corrected for the volume change before the final absorbance is subtracted from it. This correction for volume change is done as follows:

$$\text{Corrected initial absorbance} = \frac{(\text{initial absorbance})(\text{initial volume})}{(\text{final volume})}$$

Method B yields two absorbance change readings, the first measuring pyruvate and the second P-enolpyruvate. It is the most comprehensive, but in many cases it is unnecessary to know both values and would not be done as it takes extra time.

Table V. Methods for determining P-enolpyruvate concentration.

| Method   | Step | Additions to Cuvette <sup>a</sup> | Compounds Measured by Decrease in Absorbance <sup>b</sup> |
|----------|------|-----------------------------------|---|
| Method A | 1.   | Assay medium + ADP, LDH, PK       | ----  |
|          | 2.   | Sample                            | P-enolpyruvate + pyruvate                                 |
| Method B | 1.   | Assay medium, PK, sample          | ----  |
|          | 2.   | LDH                               | pyruvate  |
|          | 3.   | ADP                               | P-enolpyruvate  |
| Method C | 1.   | Assay medium, LDH, PK, sample     | ----  |
|          | 2.   | ADP                               | P-enolpyruvate  |
| Method D | 1.   | Assay medium + ADP, LDH, sample   | ----  |
|          | 2.   | PK                                | P-enolpyruvate  |

<sup>a</sup>The additions are made for a given step, then the absorbance is read in the spectrophotometer before the additions for the next step are made. The meanings of the abbreviations and approximate final concentrations in the cuvette are as follows: Assay medium = 0.05 M imidazole-HCl (pH 7.0), 0.10 M KCl, 0.01 M MgCl<sub>2</sub>, 0.16 mM NADH; ADP = 1.0 or 2.0 mM ADP; LDH = 4 units or more of lactate dehydrogenase per ml; PK = 4 units or more of pyruvate kinase per ml final volume.

<sup>b</sup>The difference, corrected for dilution by large volume additions if necessary, between the previous absorbance reading and that for the given step measures the compound listed. See text for details of calculations.

Method C quantitates P-enolpyruvate alone even when pyruvate may be present. It may not be used when ADP is in the sample solution, as is usually the case in stock solutions for kinetic studies. It may be used when pyruvate kinase is present in the sample and is therefore best for equilibrium binding studies.

Method D also quantitates P-enolpyruvate alone even when pyruvate may be present. It may not be used when pyruvate kinase is present but may be used when ADP is present in the sample. Hence, it is the best method for determining P-enolpyruvate in the stock solutions for kinetic studies.

The determination of ADP concentration is like that of P-enolpyruvate, but is not complicated by the possible presence of a contaminant that can cause a falsely high reading as in the case of pyruvate contamination of P-enolpyruvate. ADP determinations were only done to analyze the stock solutions prior to kinetic studies, and therefore a method similar to Method D for P-enolpyruvate was used.

The amount of a solution whose concentration of ADP or P-enolpyruvate is to be tested must not be so great that all the NADH in the assay medium is used up. In actual practice we have tried to limit the absorbance change to 0.4 or less in order to eliminate any possible problems that might arise from low concentrations of NADH. As an indication of the sizes of test sample that will give absorbance changes of the desired magnitude, note that 5  $\mu$ l of a 10 mM solution

will give a  $\Delta A$  of 0.31, and 50  $\mu$ l of a 1.0 mM solution also gives a  $\Delta A$  of 0.31.

### Equilibrium Binding Studies

The binding of small molecules (ligands) to protein molecules by equilibrium methods involves the measurement of the following parameters: protein concentration ( $[\text{protein}]$ ), concentration of ligand bound to the protein ( $[\text{X}]_{\text{bd}}$ ), and the concentration of free ligand ( $[\text{X}]_{\text{free}}$ ) which is in equilibrium with the protein-ligand complex. The average binding of ligand to the protein at a given concentration of free ligand is then defined as

$$\bar{v} = \frac{[\text{X}]_{\text{bd}}}{[\text{protein}]}$$

The relationship between free ligand concentration and  $\bar{v}$  for independent, identical sites is given by the following equation (Van Holde, 1971):

$$\bar{v} = \frac{n K [\text{X}]}{1 + K [\text{X}]}$$

where  $n$  is the number of binding sites for the ligand on the protein and  $K$  is a constant. Since  $n$  is the principal parameter of interest, it can be found by various plotting techniques. Deranleau (1969) states that the best plotting technique is the Scatchard plot,  $\bar{v}$  vs.  $\frac{\bar{v}}{[\text{X}]_{\text{free}}}$ , which is a plot of the binding equation rearranged in the form



$$\bar{v} = n - \frac{\bar{v}}{K [X]_{\text{free}}}$$

A straight line through the data extrapolates to  $n$ , which is the intercept on the  $\bar{v}$  axis. Another acceptable plot is  $\bar{v}$  vs.  $\log [X]_{\text{free}}$ .

Two methods were selected for the equilibrium binding studies: gel filtration and ultracentrifugation with a partition cell. Equilibrium dialysis was not selected due to our fear that the long dialysis times necessary might result in loss of substrate, denaturation of protein, or other changes due to minor side reactions. Because high concentrations of enzyme are required, any contaminating enzyme activities or side reactions catalyzed by the enzyme under study may have significant effects during the time course of the experiment. The two methods selected, gel filtration and ultracentrifugation, take less time than equilibrium dialysis but one must still be careful concerning possible changes in enzyme or ligand with time. In fact, our preparations of pyruvate kinase were found to catalyze a slow conversion of P-enolpyruvate to pyruvate in the absence of ADP. The conversion was inhibited but not eliminated by operating at 0-4° and by omitting  $\text{Mg}^{++}$  from the solutions.

### Equilibrium Binding Studies by Gel Filtration

#### Description of the Method

The gel filtration method (Hummel and Dreyer, 1962; Castellino

and Barker, 1966) utilizes a long column of gel with pore sizes large enough to allow passage of the ligand whose binding is to be studied but small enough to prevent entry of the enzyme into the gel. The column is equilibrated with a solution containing a given concentration of the ligand. The enzyme is then passed through the column, and as it passes down the column, it continually re-equilibrates with the ligand which is free to come out of the pores of the gel. If the column is long enough, the enzyme will be thoroughly in equilibrium with the given concentration of ligand by the time it emerges from the column. The effluent of the column is collected in fractions, and each fraction is assayed for enzyme and ligand. As the enzyme emerges from the column, the concentration of ligand rises above the baseline level on which the column was equilibrated, indicating the amount of ligand bound to the enzyme. If the technique is working as it should, at some time after the emergence of the enzyme from the column a depression in the ligand concentration should occur, representing the ligand that was removed from the gel by binding to the enzyme.

#### Preparation of the Gel Column

A glass column (0.8 x 120 cm) was treated with a hot 1% solution of dimethyldichlorosilane in benzene, then dried and washed with a detergent. Bio-Gel P-6 or P-10, 50 to 100 mesh, was washed with

25% methanol, then with distilled deionized water, followed by 0.5 M KCl and several volumes of imidazole-KCl buffer (0.05 M imidazole, pH 7.0, 0.1 M KCl, 0.01 M  $\text{MgCl}_2$ ). After packing the gel into the column in stages, the gel was washed at the flow rate to be used in the experiment with the desired concentration of P-enolpyruvate dissolved in the imidazole-KCl buffer. To assure complete equilibration of the gel, at least two bed volumes of the P-enolpyruvate solution were passed through the column and then the concentration of P-enolpyruvate in the effluent was assayed.

#### Operation of the Gel Column

The binding experiments were done at 4° in order to reduce a slow hydrolysis of P-enolpyruvate. In some experiments, EDTA was substituted for  $\text{MgCl}_2$  in the imidazole-KCl buffer for the same reason. This hydrolysis of P-enolpyruvate was further investigated and the methods used are discussed in a later section.

Approximately 20 mg of bovine skeletal muscle pyruvate kinase in a volume of 1 to 2 ml were dialyzed overnight against the imidazole-KCl buffer. Before applying the sample to the column, P-enolpyruvate (from a stock 0.1 M solution) was added to give a concentration in the sample equal to that at which the column had been equilibrated. The sample was then applied to the top of the column and eluted with the same P-enolpyruvate solution used for equilibration, maintaining a

flow rate in the neighborhood of 0.3 ml/min and collecting fractions of 0.5 to 1.0 ml. The fractions were first analyzed for P-enolpyruvate concentration, then for pyruvate kinase activity, and lastly for absorbance at 280 nm.

The concentration of P-enolpyruvate in the gel filtration column fractions was determined by Method A (Table V). The concentrations of pyruvate kinase in the fractions from the gel column were determined by two separate means: (1) measuring the catalytic activity; and (2) measuring the absorbance at 280 nm. The latter was done directly on the solutions without dilution by employing cells with 1 mm pathlength. These cells were filled and emptied with a blunted 6.5 cm long 20 gauge needle attached to a 1 ml syringe. The absorbances of the fractions were read from the lowest to the highest without rinsing the cells between readings. The catalytic activity determinations were done on dilutions under the standard assay conditions. The specific activity of the enzyme was determined before applying the sample to the column by measuring activity and absorbance at 280 nm of a dilution of the enzyme sample. The measurements of catalytic activity in the fractions from the gel column were converted to enzyme concentration using the specific activity. The two determinations of enzyme concentration (activity and absorbance) were usually in close but not always exact agreement. For binding calculations, the concentrations of enzyme were converted to units of mM, using a

molecular weight of 230,000 (Cardenas et al. , 1973).

The ratios of bound P-enolpyruvate to enzyme were calculated using the protein concentrations obtained from the measurements of absorbance at 280 nm for all experiments except those numbered 1 and 2, which were based on activity measurements.

#### Calculation of Average Binding of P-enolpyruvate to Pyruvate Kinase

The average concentration of P-enolpyruvate in the column effluent before emergence of the pyruvate kinase was termed the "baseline" value of P-enolpyruvate. This baseline value is the concentration of free P-enolpyruvate that is in equilibrium with the pyruvate kinase. The baseline value was subtracted from the P-enolpyruvate concentrations obtained for fractions containing pyruvate kinase to obtain the concentrations of bound P-enolpyruvate.

The average binding of P-enolpyruvate for a given experiment was then calculated by two methods, as follows:

Method 1: For each fraction in which concentration of pyruvate kinase was greater than 0.010 mM, the ratio of bound P-enolpyruvate (in mM) was calculated. The average of all such ratios was then taken.

Method 2: The sum of the concentrations of bound P-enolpyruvate was divided by the sum of the pyruvate kinase concentrations. This method is equivalent to calculating the areas of bound P-enolpyruvate and of pyruvate kinase on graphs and dividing one area by

the other. This is the method specified by Castellino and Barker (1966).

#### Equilibrium Binding Studies in the Ultracentrifuge

Equilibrium binding studies of P-enolpyruvate to bovine skeletal muscle pyruvate kinase were also carried out using partition cells in the analytical ultracentrifuge. Methods similar to those of Hirsch-Kolb *et al.* (1970), but without radioactive labels, were followed. Two types of cells were used, one with a movable partition and the other with a fixed partition. Some difficulty was experienced with the rubber springs sticking against the windows and not bringing the movable partition up at the end of the run, but with some care in insertion of the springs, either type of cell can be used with success.

Bovine skeletal muscle pyruvate kinase (14 mg/ml in experiment number 9; 5-7 mg/ml in experiments 10-13) was dialyzed overnight against 0.05 M imidazole-HCl (pH 7.0), 0.10 M KCl, and  $\text{MgCl}_2$  or EDTA as indicated in the Results section. After determination of activity and enzyme concentration, P-enolpyruvate was added to the desired level and the cell was loaded. The movable partition cell could be loaded by pushing the partition down with the end of the blunt syringe needle used for adding the sample. The fixed partition cell had to be loaded in two stages, centrifuging the cell in the rotor after the first addition to force the sample through the Whatman No. 1

filter paper placed on the partition.

The cells were centrifuged at 60,000 rpm (top speed of rotor) until the boundary in the schlieren optical system was seen to go below the partition, a period of 1 1/4 to 1 3/4 h. The centrifuge was stopped without braking. The solution was removed from the upper chamber with a syringe and blunt needle, then the gasket and plug were replaced in the filling hole, the cell was unscrewed, one window was carefully taken away, and the contents of the lower chamber were removed. Concentrations of P-enolpyruvate and the enzyme were determined in the solutions from the upper and lower chambers by the same methods used in the gel filtration binding studies. The amount of pyruvate kinase in the upper solution was usually negligible.

In order to check for sedimentation of P-enolpyruvate under the conditions employed, a solution of 0.51 mM P-enolpyruvate was centrifuged in the movable partition cell for 1 3/4 h at 60,000 rpm. The upper solution was found to have 95% of the original concentration of P-enolpyruvate, while the lower chamber had 105%. In order to correct for this sedimentation of unbound P-enolpyruvate in the binding studies, the values measured for the upper and lower solutions were adjusted in the following manner: the P-enolpyruvate concentration in the upper solution was incremented by 5%; the lower solution concentration was decremented by the same amount. The adjusted concentration of P-enolpyruvate for the upper solution (the

concentration of free P-enolpyruvate) was then subtracted from that of the adjusted lower compartment concentration to obtain the concentration of bound P-enolpyruvate. The number of P-enolpyruvate molecules bound per pyruvate kinase molecule was calculated in the same way as in Method 1 of the gel filtration binding studies.

#### Kinetics of P-enolpyruvate Phosphatase Activity of Pyruvate Kinase

Bovine or rabbit skeletal muscle pyruvate kinase was dissolved to give concentrations of 2-6 mg/ml and dialyzed overnight against 0.05 M imidazole-HCl (pH 7.0), 0.1 M KCl, 4 mM  $\text{MgCl}_2$ . Standard assays and absorbance measurements were made to determine specific activity. The solution of bovine pyruvate kinase was divided into aliquots and to each aliquot was added a different amount of 0.1 M P-enolpyruvate to give a range of concentrations from 0.10 to 2.0 mM. The samples were incubated at 25°. At intervals over 4 1/2 h, the aliquots were measured for pyruvate and P-enolpyruvate concentrations by Method B (Table V). The rabbit muscle enzyme was investigated at the single concentration of 1.0 mM P-enolpyruvate in the same manner. The velocity of P-enolpyruvate hydrolysis, at initial concentrations of 1.0 mM P-enolpyruvate, was also measured with the bovine enzyme with  $\text{Mg}^{2+}$  omitted from the dialysis buffer and incubation solution. The rate of conversion of



P-enolpyruvate was calculated both as the rate of disappearance of P-enolpyruvate and as the rate of appearance of pyruvate, with the two methods giving the same results within experimental error.

#### Homogenization of Tissue Samples for Electrophoresis

Bovine tissues other than erythrocytes were homogenized in a buffer consisting of 0.05 M Tris-HCl (pH 7.5 at room temperature), 0.1 M KCl, 10 mM  $\text{MgCl}_2$ , 10 mM  $\beta$ -mercaptoethanol. Chicken tissues were homogenized with 0.02 M Tris-HCl (pH 7.5 at room temperature), 0.15 M KCl, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol. Mouse tissues were homogenized in 0.05 M Tris-HCl (pH 7.5 at 4<sup>o</sup>), 0.5 M sucrose, 10 mM  $\beta$ -mercaptoethanol. (The mercaptoethanol was always added to the homogenization buffer on the day of use.)

Tissues were minced with razor blade or scissors before homogenization. Known weights of adult skeletal muscle or uterus were homogenized with two volumes (2 ml of buffer/g of tissue) of the buffer in a Virtis homogenizer. Known weights of other adult tissues and of the fetal tissues were homogenized with one to three volumes of the buffer in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 10,000 x g for 20 minutes, then diluted to give solutions with the desired concentration of pyruvate kinase activity. Usually the tissue extracts were diluted to 4-10 pyruvate kinase units/ml

(micromoles/min/ml). When it was desired to examine minor electrophoretic bands, the tissue extracts were diluted less or not at all.

Red blood cells (erythrocytes) were obtained from heparinized blood by centrifuging for 5 to 10 min at 3,000 x g, then aspirating off the plasma and buffy coat. The erythrocytes were then washed three to five times by suspending the cells, either in 1% NaCl for bovine erythrocytes or Earle's basic saline solution (Earle, 1943) for chicken erythrocytes, and recentrifuging. The cells were then lysed by adding an equal volume of 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol to the final pellet. After lysis, red cell ghosts were removed by centrifugation for 10 min at 3,000-10,000 x g.

Bovine leucocytes were separated from contaminating erythrocytes by several centrifugations of the buffy coat for 5 min at 3,000 x g. The leucocytes were washed twice with 1% NaCl, then suspended in an equal volume of the homogenization buffer used for bovine tissues and lysed by sonication. Cell debris was removed by centrifuging 20 min at 10,000 x g.

All homogenizations and centrifugations were performed at 0-4°. Tissue extracts were dialyzed for 2 h in the appropriate electrophoresis buffer before sample application. Electrophoretic analysis was performed on all samples at least once before freezing, but many of the electrophoretic patterns shown in this thesis were

obtained using frozen extracts. No differences between fresh and frozen extracts were observed. However, loss of activity was often observed if extracts were frozen without containing 0.5 M sucrose. Consequently, only tissue extracts that had been dialyzed against electrophoresis buffer were frozen for re-use.

### Electrophoresis of Pyruvate Kinase on Cellulose Acetate

#### Buffers

The basic buffer was that of Susor and Rutter (1971) consisting of 0.02 M Tris-HCl, 0.5 M sucrose and 10 mM  $\beta$ -mercaptoethanol. The pH varied from pH 7.0 at room temperature (approximately pH 7.5 at 4°) to pH 7.5 at room temperature (approximately pH 8.0 at 4°). The higher pH was usually employed for long running times of samples with bands that migrated toward the cathode--for example, chicken tissues and those bovine tissues where good K-M separation was required. The higher pH kept the cathodic bands from migrating off the end of the strip during the long running times necessary to achieve separation of the isozymes. In contrast, the lower pH was employed for short running times of the bovine tissues, when it was desired to have the cathodic K and M isozymes move well off the origin.

Certain additions were made to the above basic buffer: (1) 1 mM fructose 1,6-diphosphate was added whenever a separation of K and M

isozymes was desired, which was the case in most of the electrophoretic studies reported in this thesis. (2) 1 mM EDTA was added in many instances in order to increase the stability of the pyruvate kinase, and was almost always used in the later work with bovine tissues. (3) 4 mM  $\text{MgCl}_2$  was added to the basic electrophoresis buffer in addition to fructose 1,6-diphosphate for electrophoresis of all chicken tissues.

### Methods

Gelman cellulose acetate strips (Sephacel III, 1 x 6 in.) were soaked for at least 30 min in electrophoresis buffer containing fraction V bovine serum albumin (1 mg/ml) as suggested by Susor and Rutter (1971). The strips are placed flat on the surface of the buffer to facilitate uptake of the buffer. Occasionally a batch of the strips seemed resistant to wetting, sometimes trapping small pockets of air within the membranes and rendering them unsuitable for use. This problem could be alleviated in many cases by warming the buffer to room temperature. In extreme cases, the suggestion of the manufacturer to include 0.5% ethanol in the buffer or floating the strips initially on distilled water and transferring them to the buffer proved effective.

Immediately after application of the sample, the strip was placed on Whatman No. 1 paper which was soaked with buffer, and

the strip was blotted until surface moisture was removed, being careful not to dry the strip.

A Gelman serum applicator was used to place the solution on the cellulose acetate strip. The applicator, which has a capacity of approximately 3  $\mu$ l, was loaded at its center using capillary pipettes. A Gelman dual applicator was sometimes used to apply two samples side-by-side on a single strip, in order better to compare their electrophoretic mobilities. Replicate applications were used for low activity extracts.

The electrophoresis was carried out at 200-250 volts and 4° and for running times varying from 4 to 35 h depending on the resolution required. For electrophoresis of bovine tissues in which it was desired to see type L<sub>4</sub> pyruvate kinase, 4 h (at 250 volts) was the longest time permissible without danger of running the type L<sub>4</sub> off the end of the cellulose acetate strip. On the other hand, at least 20 h (at 200 volts) was required to separate bovine type K<sub>4</sub> and type M<sub>4</sub> pyruvate kinase sufficiently to see the individual K-M hybrids clearly. In the bovine electrophoresis, the longer running times were usually done at 200 volts rather than 250 volts in order to minimize the drying out of the cellulose acetate due to heating effects. On the other hand, although 16 h was required to separate chicken type K from type M, all of the chicken electrophoresis was run at 250 volts without any problems due to drying of the cellulose acetate.

## Detection

The method used to visualize the pyruvate kinase bands after electrophoresis was that devised by Susor and Rutter (1971) with the modifications of Cardenas and Dyson (1973). The method involves the placing of the strips on a thin film of agar containing the components of the lactate dehydrogenase-coupled assay system for pyruvate kinase. Under long-wave ultraviolet light, the NADH in the assay system fluoresces except in regions where pyruvate kinase is present and has caused the NADH concentration to decrease. Thus the bands of pyruvate kinase appear as dark regions. Contact photographs may be made under ultraviolet light, resulting in exposure of the photographic medium only in regions of NADH loss.

The methods of Cardenas and Dyson (1973) were used for detection of pyruvate kinase after electrophoresis with only the following few exceptions. The photographs of the chicken electrophoretic patterns were taken mostly with Kodak Spectrum Analysis No. 1 plates or with Kodak Electron Image plates. The exposure times were 4-6 sec and 3-4 sec, respectively, pointing the UVL-21 lamp (Blak-Ray) at the wall or ceiling above the strips. The mouse electrophoresis photos and some of the bovine photos were also taken with these plates. However, most of the bovine pictures were taken with Kodak Kodabromide F-5 paper as originally suggested by Susor and Rutter (1971).

For photography with the Kodabromide F-5 paper, a box was constructed of light cardboard to hold the UVL-21 lamp. The ultraviolet light from the lamp was allowed to pass through a 2 cm-diameter hole over which was placed a Kodak Wratten No. 18A filter (maximum transmittance at 340 nm). The box was mounted on a stand 0.5 m above the strips, and the photography paper was exposed for 2-4 sec with direct light.

The detection of bands of low activity was facilitated by warming the combined cellulose acetate strip and agar film (containing the detection solution) in a closed chamber above water at 40-60°.

### Kinetic Studies

The velocity of chicken and bovine type K<sub>4</sub> pyruvate kinase isozymes at differing concentrations of substrates was studied using the same lactate dehydrogenase-coupled assay system already described. The assay medium consisted of 0.05 M imidazole-HCl (pH 7.5 at 25°), 0.10 M KCl, 10 mM MgCl<sub>2</sub>, 0.16 mM NADH, approximately four units of lactate dehydrogenase per ml, and varying concentrations of ADP and P-enolpyruvate as indicated in the Results section. Fructose 1,6-diphosphate, when present, was 1.0 mM. The total volume of each assay was 1.0 ml in cuvettes of 4 mm sample width and 10 mm pathlength. The decrease in absorbance at 340 nm was recorded on the Beckman Acta III spectrophotometer,

which was equipped with an automatic sample changer. The temperature was maintained at  $25^{\circ}$ , and each set of assays was placed into the spectrophotometer or into an aluminum block in contact with the circulating water for at least 4 min immediately prior to starting the reaction in order to insure complete temperature equilibration.

Assays were initiated by adding an aliquot of the enzyme preparation. The amount of enzyme to be added was chosen such that the maximal velocity would be approximately 0.03 absorbance units per min for hyperbolic kinetic profiles and approximately 0.15 absorbance units per min for sigmoidal velocity profiles. These rates could not be exceeded without the occurrence of non-linear initial velocities at the lower substrate concentrations.

For kinetic studies of the pyruvate kinase in adult chicken liver and fetal chick thigh muscle, the tissue extracts, obtained as for electrophoretic analysis, were centrifuged for 30 min at  $100,000 \times g$  and then either (a) passed through a  $1.5 \times 3.0$  cm column of Sephadex G-25 equilibrated with 0.05 M imidazole-HCl (pH 7.5), 0.10 M KCl, 10 mM  $MgCl_2$ , and 10 mM  $\beta$ -mercaptoethanol, or (b) dialyzed against the same buffer for 16 h. Extracts prepared by the two procedures gave the same results. For one kinetic study, saturated ammonium sulfate was added to the centrifuged extracts to bring them to 90% of saturation. The precipitate was collected by centrifugation, washed with 90% saturated ammonium sulfate, dissolved in and dialyzed



against the G-25 column buffer for 2 h. For another kinetic study, an extract of 11-day fetal chick thigh muscle was put through the G-25 column as before, then incubated at 37° for 30 min in the presence of 33 mM EDTA, as done by Walker and Potter (1973), in order to determine whether the kinetics could be converted to a different pattern, as seen with some extracts of rat tissues (Pogson, 1968a, b; Walker and Potter, 1973).

Using the coupled assay system, extracts of adult chicken liver had appreciable activity when fructose 1,6-diphosphate was added without P-enolpyruvate, presumably due to utilization of fructose 1,6-diphosphate by other glycolytic enzymes in the extracts. Therefore, blank assays containing all components except P-enolpyruvate were run simultaneously with the liver assays. The absorbance values of sample and blank were automatically recorded by means of a teletype print-out, installed as a custom-built modification on the Acta III spectrophotometer. The blank readings were subtracted from the sample readings, and the net readings were plotted as a function of time to determine the rate of pyruvate kinase activity. (This procedure was adopted instead of running the sample against the blank in double-beam mode in order to monitor activity of the blank.)

The activity of enolase (E. C. 4.2.1.11) or other enzymes causing the disappearance of P-enolpyruvate was measured by monitoring the P-enolpyruvate concentration at 240 nm in an assay medium

consisting of 0.05 M imidazole-HCl (pH 7.5), 0.10 M KCl, 10 mM  $\text{MgCl}_2$ , and varying concentrations of P-enolpyruvate. The rate of disappearance of P-enolpyruvate was calculated using an extinction coefficient of  $1400 \text{ M}^{-1} \text{ cm}^{-1}$  (see Wold and Ballou, 1957).

Kinetic constants for hyperbolic curves were obtained by plotting the data in double-reciprocal form and fitting with a weighted least squares straight line. Maximal velocities for sigmoidal kinetics in chicken extracts were obtained by extrapolating plots of  $1/v$  vs.  $1/S^2$  to infinite substrate concentration. For the sigmoidal bovine  $K_4$  pyruvate kinase kinetics, maximal velocities were obtained by finding the value of  $n$  which gave the best least squares computer fit of a plot of  $1/v$  vs.  $1/S^n$ ; the maximal velocity was then obtained from the intercept on the  $1/v$  axis. These values for the maximal velocities were used for calculation of the Hill plots.

### Immunological Studies

Testing of immunological cross-reactivity was done by titrating constant amounts of samples of pyruvate kinase (chicken tissue extracts or bovine type  $K_4$  preparations) with increasing quantities of antiserum made against bovine or chicken type M pyruvate kinase, prepared in rabbits by the methods described by Cardenas et al. (1973). The tests for cross-reactivity were carried out in a solution consisting of 0.05 M potassium phosphate (pH 7.0), 0.8% NaCl, and

2 mM  $\beta$ -mercaptoethanol. The solutions were incubated at 37° for 1 h, then at 4° for 5 h, and centrifuged 30 min at 4,000  $\times$  g before assaying the supernatants for the remaining pyruvate kinase activity.

#### Partial Purification of Bovine Type K<sub>4</sub> Pyruvate Kinase

Bovine type K<sub>4</sub> pyruvate kinase was prepared from kidney by a modification of the procedure used for purification of type M<sub>4</sub> pyruvate kinase from skeletal muscle (Cardenas et al., 1973). All steps were carried out at 0-4°.

Step 1: Extraction - Fresh kidney or partially thawed frozen kidney, from which as much fat as possible had been trimmed, was passed through a pre-cooled meat grinder. The ground kidney was homogenized at high speed in an Osterizer food blender for 30 s with a buffer solution consisting of 0.05 M Tris-HCl (pH 7.5 at room temperature), 0.15 M KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 10 mM  $\beta$ -mercaptoethanol (2 ml of buffer solution per g of kidney). The mixture was stirred for one hour and then centrifuged at 8,000  $\times$  g for 20 min. The supernatant was filtered through several layers of cheesecloth and a loose plug of glass wool.

Step 2: Heat Treatment - To the filtered supernatant from Step 1 was added enough 1.0 M imidazole (pH 7.0) to make it 0.02 M in imidazole. The pH was checked and adjusted to 7.0 if it was more than 0.3 units off. The solution was then heated rapidly to 60° by

swirling in an 80-85<sup>o</sup> water bath, maintained at 60<sup>o</sup> for 4 min with continued swirling, and then cooled to 10<sup>o</sup> by swirling in an ice-water bath. This step was carried out in one-liter erlenmeyer flasks with no more than 500 ml of extract at a time. The resulting suspension was centrifuged at 10,000 x g for 20 min and the precipitate was discarded.

Step 3: Ammonium Sulfate Fractionation - The supernatant from the heat treatment was brought to 46% ammonium sulfate saturation at 0<sup>o</sup> by the gradual addition with stirring of 270 g solid ammonium sulfate per liter of solution. After stirring for 30 min after the final addition of ammonium sulfate, the mixture was centrifuged for 20 min at 10,000 x g. The supernatant was then brought to 60% ammonium sulfate saturation by the addition of 90 g of solid ammonium sulfate per liter of 46% saturated solution. After centrifuging for 20 min at 10,000 g, the precipitate was saved and suspended at a concentration of 20 to 40 mg per ml in 80% saturated ammonium sulfate containing 10 mM  $\beta$ -mercaptoethanol. This suspension was stable for several weeks when stored at 0-4<sup>o</sup>.

Step 4: Carboxymethyl Sephadex Chromatography - Carboxymethyl Sephadex C-50 was allowed to swell in deionized water for 48 h, then washed in a Büchner funnel with several volumes of 1 M KCl, then with 0.1 M KOH, next with 0.1 M HCl, and finally with the buffer solution to be used in the experiment, rinsing with distilled

deionized water between each step. (CM-Sephadex gel that had been used for chromatography was cleaned for re-use by this same procedure, except that the gel was allowed to sit in the 0.1 M KOH and 0.1 M HCl for one half-hour each before continuing with the procedure.) The CM-Sephadex was then suspended in the buffer and titrated with 6 M KOH until the pH of the gel suspension was within 0.2 units of the pH of the buffer. The gel was next allowed to settle several times, pouring off the supernatant and adding more buffer with stirring each time.

The buffer used for the CM-Sephadex chromatography consisted of 0.04 M imidazole-HCl (pH 6.5 at 4°), 0.5 M sucrose, 1 mM EDTA, and 10 mM  $\beta$ -mercaptoethanol. Higher pH, up to pH 8.0 at 4°, was also used for several columns.

The prepared gel was poured in sections as a slurry (total volume about two times the volume of the settled gel) to form a column 1.5 x 35-40 cm. The column was further equilibrated by passing at least two bed volumes of buffer through the column at the flow-rate to be used in the experiment and checking to see that the pH of the effluent was identical to that of the equilibration buffer.

After the equilibration of the column, 200 to 500 mg of protein from the 60% ammonium sulfate precipitate (the results of Step 3) were dialyzed 4 h against the equilibration buffer in 1/4 inch diameter dialysis tubing and layered onto the column. The flow-rate of 25-30

ml was maintained constant by means of a peristaltic pump. After approximately 100 ml of buffer had been passed through the column, a one liter linear gradient of 0-0.3 M KCl in the buffer was used for elution. Fractions of 4-5 ml volume were collected and assayed for pyruvate kinase activity. The fractions containing highest pyruvate kinase activity were combined as appropriate and dialyzed for several hours against saturated ammonium sulfate solutions (pH 7.0) containing 10 mM  $\beta$ -mercaptoethanol. Typical elution patterns for the CM-Sephadex chromatography are shown in the Results section.

#### Dissociation and Reassociation of Hybrid Isozymes

Two isozymes of intermediate electrophoretic mobility from bovine kidney, identified by their electrophoretic positions as  $K_2L_2$  and  $K_3M$ , were obtained as a by-product of the  $K_4$  isolation.  $K_2L_2$  was eluted from the CM-Sephadex column by the buffer wash as a second peak of pyruvate kinase activity after a mixed first peak containing  $L_4$  and  $KL_3$ . (The column from which  $K_2L_2$  was obtained was actually loaded with 1200 mg of protein, somewhat over twice the normal maximum load.) The  $K_2L_2$  was then chromatographed on a 1.5 x 10 cm column of DEAE-cellulose in 10 mM potassium phosphate (pH 7.0), 0.5 M sucrose, 2 mM dithiothreitol. A linear gradient of 0-0.25 M KCl in this buffer in a total volume of 300 ml was used to elute the  $K_2L_2$ . Approximately 1.5 mg of protein containing  $K_2L_2$ ,

with a specific activity for pyruvate kinase of 2.2, was obtained from this column.

$K_3M$  was eluted somewhat after  $K_2M_2$  and a little before the  $K_4$  in the gradient on the CM-Sephadex column. Some  $K_3M$  could be obtained essentially free of  $K_2M_2$  and  $K_4$  by selection of the right fractions.

The isolated  $K_2L_2$  and  $K_3M$  were each then dissociated in 3.5 M guanidine-HCl and reassociated by dilution and dialysis according to the methods that were used for hybridization of types  $M_4$  and  $L_4$  pyruvate kinase by Cardenas and Dyson (1973).

#### Isoelectric Focusing

Isoelectric focusing of purified bovine skeletal muscle pyruvate kinase and of partially purified bovine kidney type  $K_4$  pyruvate kinase was carried out in a 110 ml capacity LKB isoelectric focusing apparatus at 4°. LKB ampholines with a pH range of 7 to 10 were employed. The voltage (anode at the top of the column) was gradually raised to 600 volts and maintained for 48-60 h. The apparatus was emptied at a flow-rate of 1.0 to 1.5 ml/min into 2.0 ml fractions. Each fraction was assayed for pyruvate kinase activity and absorbance at 280 nm. The pH of selected fractions was measured, leaving the electrode in the solution to be measured for about 8 min before recording to compensate for slow equilibration with the ampholines.

The pH meter used was a Radiometer Model 26 (with an expanded scale) equipped with a small-size combination electrode.



## RESULTS

### Extinction Coefficient of Bovine Skeletal Muscle Pyruvate Kinase

From the dry weight determination, we find that a 1 mg/ml solution of bovine skeletal muscle pyruvate kinase has an absorbance at 280 nm of 0.55 per cm of lightpath and produces 3.88 interference fringes in a 12-mm synthetic boundary cell. The ratio of absorbance at 280 nm to that at 260 nm is 1.71. The value for the absorbance at 280 nm was used in the calculation of concentration of purified bovine skeletal muscle pyruvate kinase.

### P-enolpyruvate Phosphatase Activity of Skeletal Muscle Pyruvate Kinase

The kinetics of hydrolysis of P-enolpyruvate to pyruvate, carried out at 25° without the addition of ADP, by preparations of bovine skeletal muscle pyruvate kinase are shown in Fig. 1 on a double-reciprocal (Lineweaver-Burke) plot. The Michaelis constant ( $K_m$ ) for P-enolpyruvate is 0.23 mM, compared to 0.04 mM for the ordinary pyruvate kinase reaction when ADP is present. The maximal velocity obtained from the  $1/v$  intercept is  $2.3 \times 10^{-4}$  micromoles P-enolpyruvate per min per mg enzyme. Since the maximal velocity when ADP is present at 2.0 mM is about  $2.3 \times 10^2$ , the velocity of the P-enolpyruvate hydrolase activity is a million times less than

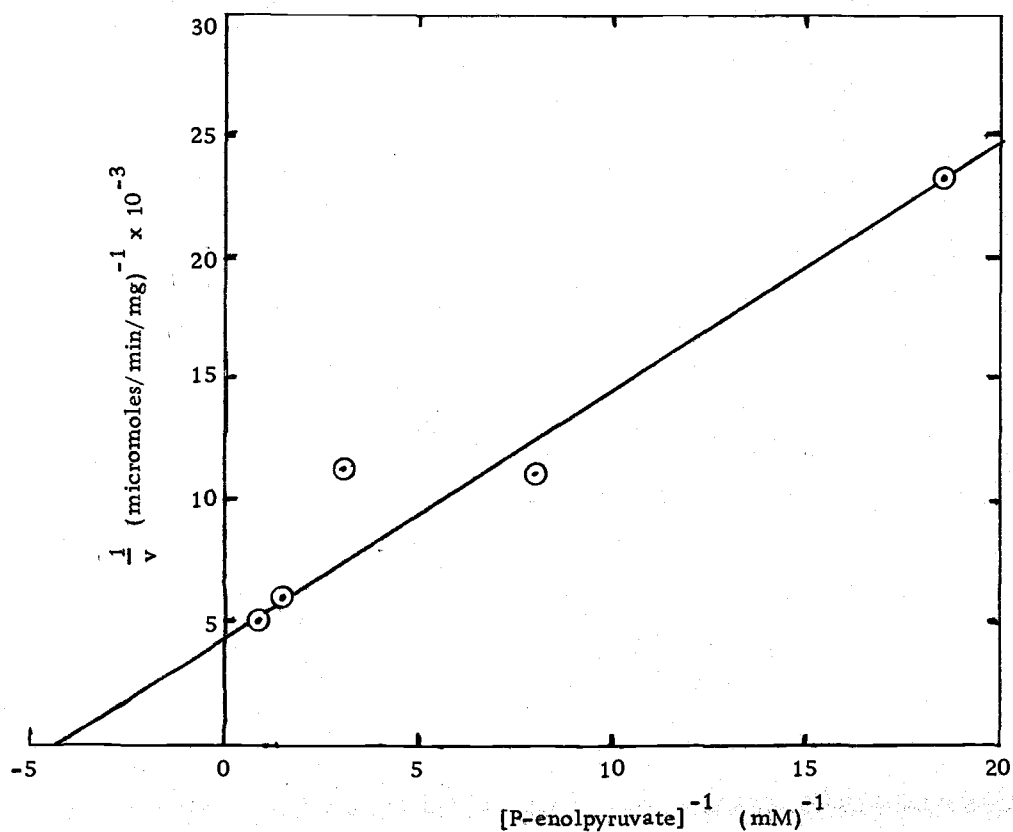


Fig. 1. Double-reciprocal (Lineweaver-Burke) plot of the hydrolysis of P-enolpyruvate by bovine skeletal muscle pyruvate kinase in the absence of ADP at 25°. The assay medium contained 0.05 M imidazole-HCl (pH 7.0), 0.1 M KCl, and 4 mM MgCl<sub>2</sub>. The  $K_m$  for P-enolpyruvate is 0.23 mM and the maximal velocity is  $2.3 \times 10^{-4}$  micromoles of P-enolpyruvate hydrolyzed per min per mg of enzyme.

the ordinary pyruvate kinase reaction.

At an initial P-enolpyruvate concentration of 1.0 mM and with 1 mM EDTA present instead of 4 mM  $\text{MgCl}_2$ , the rate of P-enolpyruvate hydrolysis at  $25^\circ$  in the presence of bovine skeletal muscle pyruvate kinase was approximately one-fourth of that measured in the presence of 4 mM  $\text{MgCl}_2$ .

The commercial preparation of rabbit skeletal muscle pyruvate kinase also carried out the hydrolysis of P-enolpyruvate in the absence of ADP. At 1.0 mM P-enolpyruvate the rate of hydrolysis was  $7 \times 10^{-4}$  micromoles/min/mg enzyme.

#### Equilibrium Binding Studies of P-enolpyruvate to Bovine Skeletal Muscle Pyruvate Kinase

Sample elution profiles from gel filtration columns used for the binding studies with bovine skeletal muscle pyruvate kinase are illustrated in Figs. 2 and 3. Both figures show experiments carried out at  $4^\circ$  in order to minimize hydrolysis of P-enolpyruvate. In Fig. 2 (Expt. 6, Table VI) the buffer solution contained 10 mM  $\text{MgCl}_2$ , whereas in Fig. 3 (Expt. 7, Table VI) 0.10 mM EDTA was substituted for  $\text{MgCl}_2$ . When  $\text{MgCl}_2$  was used instead of EDTA, there was an unexpected drop in P-enolpyruvate eluted immediately after the enzyme, due no doubt to P-enolpyruvate phosphatase activity. In the presence of EDTA, used to reduce this activity, the theoretically

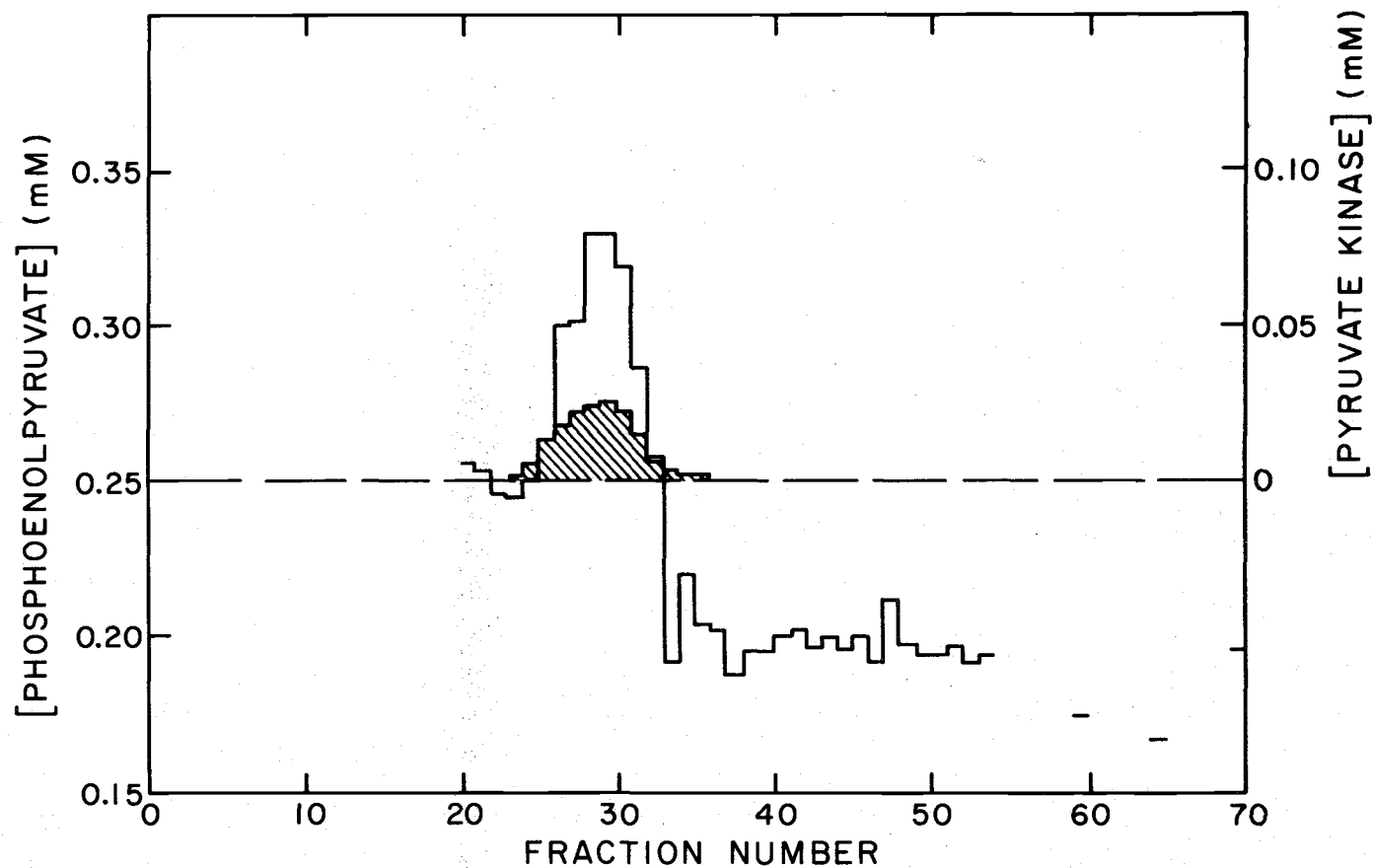


Fig. 2. Elution profile of gel filtration column used in equilibrium binding study experiment number 6. The column was equilibrated on 0.25 mM P-enolpyruvate in the presence of 4.0 mM  $\text{MgCl}_2$ . The temperature was  $4^\circ$ , and the volume of each fraction was 0.75 ml. The shaded area represents the concentration of pyruvate kinase.

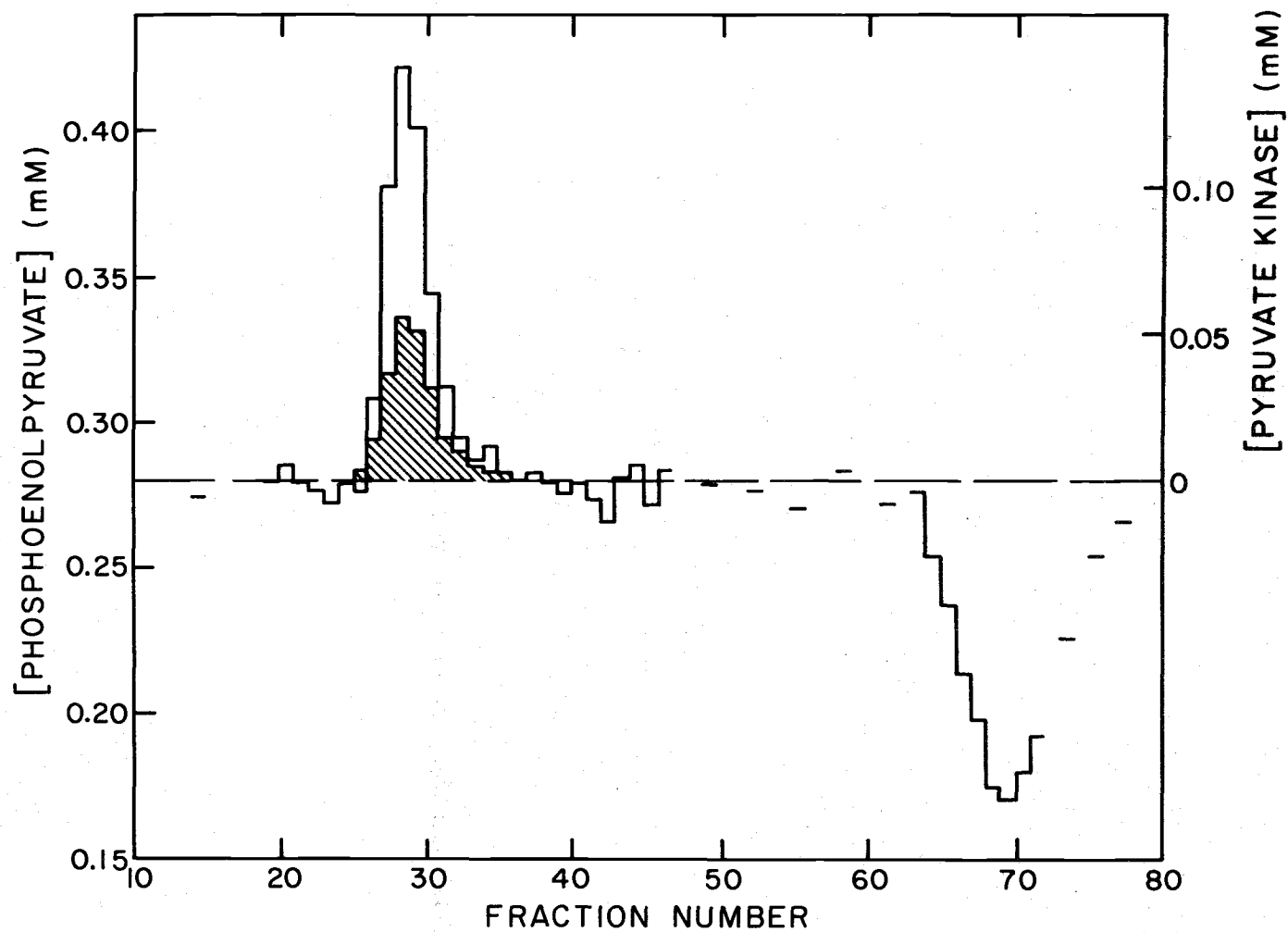


Fig. 3. Elution profile of gel filtration column used in equilibrium binding study experiment number 7. The column was equilibrated on 0.28 mM P-enolpyruvate in the presence of 0.10 mM EDTA. The temperature was 4°, and the volume of each fraction was 0.6 ml. The shaded area represents the concentration of pyruvate kinase.

Table VI. Binding of P-enolpyruvate to bovine skeletal muscle pyruvate kinase.<sup>a</sup>

| Experiment Number | Conditions <sup>b</sup>                        | [P-enolpyruvate]<br>(mM) | $\frac{[\text{P-enolpyruvate}]}{[\text{Enzyme}]}$ |                       |
|-------------------|--|--------------------------|---|-----------------------|
|                   |  |                          | Method 1 <sup>c</sup>                             | Method 2 <sup>c</sup> |
| 1                 | P-6 at 23°, 4.0 mM Mg <sup>2+</sup>            | 0.46                     | 3.2   | 2.9                   |
| 2                 | P-6 at 23°, 4.0 mM Mg <sup>2+</sup>            | 0.46                     | 2.8   | 3.2                   |
| 3                 | P-6 at 23°, 4.0 mM Mg <sup>2+</sup>            | 0.51                     | 3.6   | 3.4                   |
| 4                 | P-10 at 23°, 4.0 mM Mg <sup>2+</sup>           | 0.50                     | 2.2   | 2.3                   |
| 5                 | P-10 at 4°, 4.0 mM Mg <sup>2+</sup>            | 0.50                     | 3.8   | 3.3                   |
| 6                 | P-10 at 4°, 4.0 mM Mg <sup>2+</sup>            | 0.25                     | 2.8   | 2.4                   |
| 7                 | P-10 at 4°, 0.10 mM EDTA                       | 0.28                     | 2.3   | 2.4                   |
| 8                 | P-10 at 4°, 0.05 mM EDTA                       | 0.61                     | 3.6   | 3.4                   |
| 9                 | Ultracentrifuge at 4°, 4.0 mM Mg <sup>2+</sup> | 0.35                     | 2.4   |                       |
| 10                | Ultracentrifuge at 4°, 4.0 mM Mg <sup>2+</sup> | 0.33                     | 2.5   |                       |
| 11                | Ultracentrifuge at 4°, 0.10 mM EDTA            | 1.0                      | 4.0   |                       |
| 12                | Ultracentrifuge at 20°, 0.10 mM EDTA           | 0.17                     | 1.8   |                       |
| 13                | Ultracentrifuge at 20°, 0.34 mM EDTA           | 0.34                     | 3.3   |                       |

<sup>a</sup>The experiments are described more completely under "Materials and Methods."

<sup>b</sup>P-6 and P-10 refer to Bio-Gel polyacrylamide particles.

<sup>c</sup>See the Materials and Methods section for a description of the calculation methods. Method 2 is the more applicable method.

predicted P-enolpyruvate elution profile is seen (Castellino and Barker, 1966).

The results of binding studies carried out by the gel filtration method on bovine skeletal muscle pyruvate kinase under various conditions are presented in Table VI. Both methods for calculating the average binding are reported, but the values obtained with Method 2 are more applicable (Castellino and Barker, 1966). Also listed in Table VI are the results of binding studies carried out in the ultracentrifuge. The values were obtained by correcting for the sedimentation of unbound P-enolpyruvate, as described in the Materials and Methods section. In agreement with the binding studies performed by gel filtration, the ultracentrifuge results showed that up to four molecules of P-enolpyruvate are bound per molecule of enzyme, suggesting the presence of one binding site per subunit. The data, however, are not extensive enough to warrant calculation of a binding constant.

#### Electrophoresis of Bovine Pyruvate Kinase Isozymes

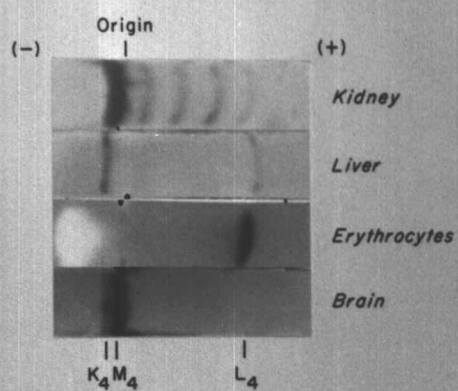
In Fig. 4 are shown the results of 4 h of cellulose acetate electrophoresis of the pyruvate kinase isozymes in several bovine tissues. Type  $L_4$  pyruvate kinase, which is found in kidney, liver, and erythrocytes, moved several centimeters toward the anode. Intermediate bands, which appear to be K-L hybrids, are seen between  $K_4$  and  $L_4$

Fig. 4. Patterns of pyruvate kinase isozymes in bovine tissues after 4 h of electrophoresis on cellulose acetate strips.

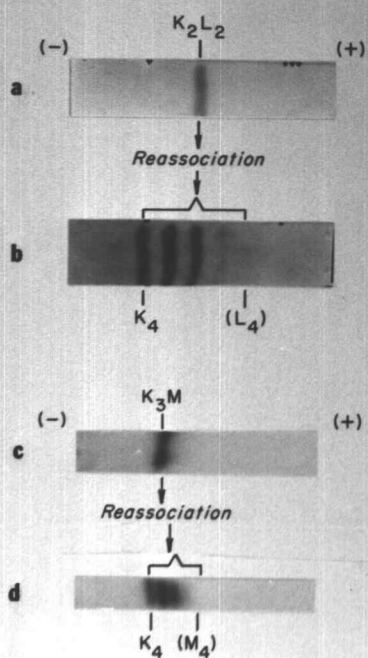
Fig. 5. Patterns of pyruvate kinase isozymes in bovine tissues after 20-35 h of electrophoresis. The embryo was taken at approximately 1 1/2-2 months gestation. The pregnant uterus was at about 5 months gestation.

Fig. 6. Results of dissociation in guanidine-HCl and reassociation of intermediate species (hybrids) of pyruvate kinase.  $K_2L_2$  before (a) and (b) after dissociation-reassociation and 4 h electrophoresis.  $K_3M$  before (c) and (d) after dissociation-reassociation and 25 h electrophoresis.

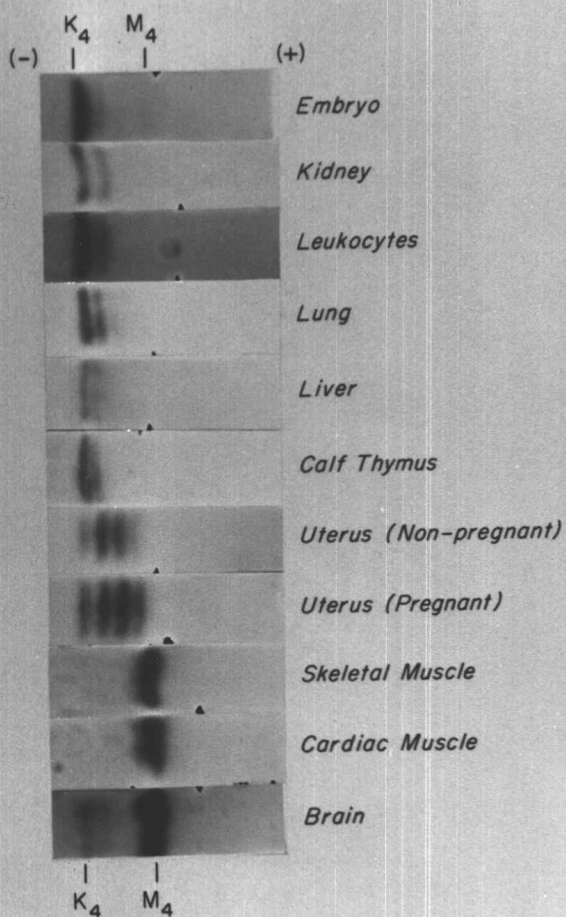




4



6



5

in kidney. Note that the  $K_4$  and  $M_4$  isozymes were barely resolved.

Electrophoresis for 20 h or more is required to separate the  $K_4$  and  $M_4$  isozymes sufficiently to see intermediate bands (Fig. 5). In agreement with previous work on other mammals, type  $M_4$  pyruvate kinase was the dominant isozyme in skeletal muscle, heart muscle, and brain. Type  $K_4$  pyruvate kinase was present in kidney, liver, lung, leukocytes, uterus, thymus, and to a small extent in brain--  
i. e., in all adult tissues examined except skeletal muscle and cardiac muscle. However, in each case  $K_4$  was accompanied by a significant amount of one or more intermediate bands between  $K_4$  and  $M_4$ .

An embryo, estimated at 1 1/2 to 2 months gestation, contained predominantly type  $K_4$  pyruvate kinase (Fig. 5) but also had small amounts of  $K_3M$ ,  $K_2M_2$ , and  $M_4$ . This result is not inconsistent with the hypothesis, advanced by Imamura and Tanaka (1972), that type K pyruvate kinase is a fetal prototype and that the M and L types are differentiated forms of pyruvate kinase, since even at 1 1/2-2 months histologically identifiable striated muscle, which may at that early stage already be in the process of a K to M shift, is already present.

#### Dissociation and Reassociation of Hybrid Isozymes

Bovine kidney showed intermediate bands both between the  $K_4$  and  $L_4$  isozymes and between  $K_4$  and  $M_4$ , suggesting the presence of two hybrid sets and thus a total of nine electrophoretic species in this

one organ. Six of these nine species are readily visible in the electrophoretic patterns of kidney presented in Figs. 4 and 5. A seventh band, corresponding to  $K_2M_2$ , has been seen in some zymograms of kidney but cannot be detected in the photograph selected for Fig. 5.

Isolation of the middle band between  $K_4$  and  $L_4$  (the presumptive  $K_2L_2$ ), followed by dissociation and reassociation, yielded a pattern similar to the original kidney K-L hybrid set (Fig. 6b). The lack of symmetry in the pattern can be explained by the greater instability of the type L isozyme. Thus this result can be interpreted as a confirmation of the subunit composition of the isolated  $K_2L_2$ .

A similar experiment was performed with one of the intermediate bands between the  $K_4$  and  $M_4$  positions. In this case the band immediately adjacent to  $K_4$  (the presumptive  $K_3M$ ) was isolated, since it is fairly abundant. Dissociation and reassociation resulted in the electrophoretic pattern shown in Fig. 6d. The statistically expected (binomial) distribution from three type K subunits and one type M subunit is 32%  $K_4$ , 41%  $K_3M$ , 21%  $K_2M_2$ , 6%  $KM_3$ , and 0.4%  $M_4$ , a result that is qualitatively consistent with the electrophoretic pattern presented.

#### Electrophoresis of Chicken Pyruvate Kinase Isozymes

Figs. 7-9 contain representative photographs of chicken pyruvate kinase patterns after 16 hr of electrophoresis at pH 8.0. Since

electrophoretic mobilities varied somewhat from run to run (due, for example, to small variations in temperature or pH of the buffer), pyruvate kinase from adult brain was used as a reference in all experiments. The zymograms in Figs. 7-9 have been aligned with the adult brain zymogram rather than according to electrophoretic origin, in order to compensate for day-to-day experimental variations.

Only two electrophoretic forms of pyruvate kinase appear to be present in chicken tissues. While both of these pyruvate kinases move toward the cathode at pH 8.0, they are clearly and reproducibly distinguishable and do not seem to be interconvertible. No additional electrophoretic forms were found when electrophoresis was carried out for 2 to 4 hr in order to detect any rapidly migrating or unstable bands of pyruvate kinase. However, in the absence of  $\text{MgCl}_2$ , an additional, but minor, rapidly migrating cathodic band was sometimes seen in zymograms of muscle extracts. The identity of this band has not been determined, but it quite possibly is an electrophoretic variant of the chicken muscle isozyme. Its high cathodic mobility may indicate a lack of binding of fructose 1,6-diphosphate to this form in the absence of  $\text{Mg}^{2+}$ . Such a phenomenon would be similar to the existence of two types of rat liver pyruvate kinase that differ in the number of fructose 1,6-diphosphate molecules bound (Hess and Kutzbach, 1971).

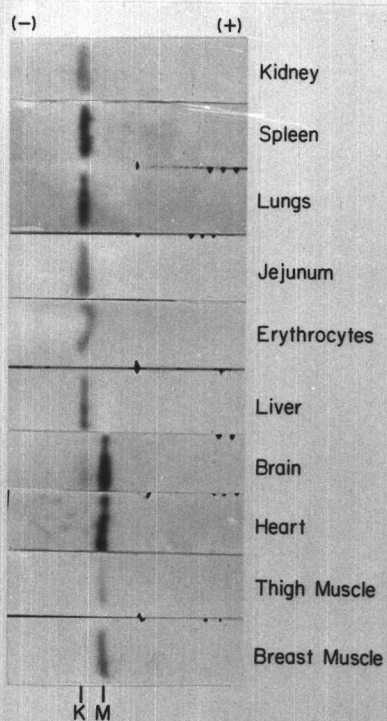
Fig. 7 shows the distribution of the two isozymes of pyruvate

Fig. 7. Patterns of pyruvate kinase isozymes in adult chicken tissues after 16 h of electrophoresis. The centrally located dark triangles mark the origins; other notches were used for identification of samples. The zymograms in this and subsequent figures were aligned using adult brain as a standard, as explained in the text.

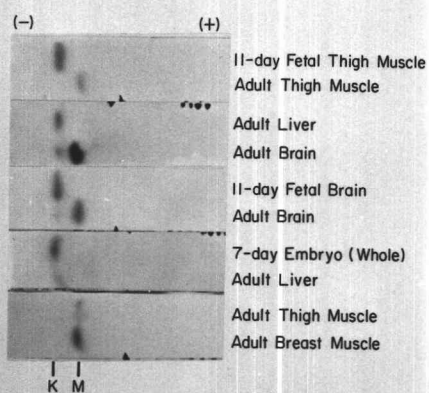
Fig. 8. Pyruvate kinase zymograms of various adult and fetal chicken tissues, run two per cellulose acetate strip.

Fig. 9. Zymograms of muscle and brain in the developing chick embryo. The time after start of incubation is given in days. Hatching occurs at 20-21 days.

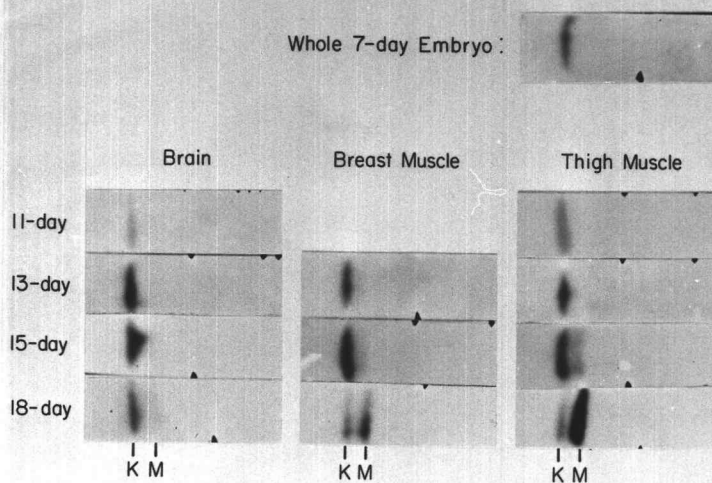
Fig. 10. Electrophoresis of adult chicken liver pyruvate kinase in various buffers. The running time was 3.0 h at 250 volts. Each buffer contained 0.5 M sucrose and 0.01 M  $\beta$ -mercaptoethanol, plus the following: (A) 0.05 M glycine-KOH, pH 10.0, (B) 0.02 M potassium phosphate, pH 7.0, (C) standard buffer without fructose 1,6-diphosphate, and (D) 0.02 M Tris-maleate, pH 5.5.



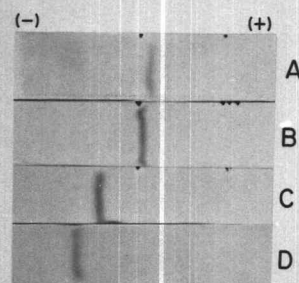
7



8



9



10

kinase in various adult tissues, while Fig. 8 presents comparative electrophoresis of extracts of selected adult and embryonic tissues. Since one of the isozymes was found only in striated muscle and brain, while the other was detected in all tissues examined except striated muscle, it seems reasonable to follow the same nomenclature used previously in mammals and to designate the isozymes as type M and type K, respectively. As will be discussed, there are many points of similarity between chicken and mammalian M and between chicken and mammalian K.

Type M, the more slowly migrating pyruvate kinase in chicken tissues, is found in breast muscle (pectoralis major), thigh muscle (femorotibialis), and heart ventricular muscle. The faster migrating pyruvate kinase, type K, is the only isozyme seen in spleen, lungs, erythrocytes, kidney, liver, and jejunum. Brain contains both isozymes, type K being the minor component.

In the early stages of development, fetal chicken tissues contain only type K, as illustrated by the patterns shown in Fig. 9 for whole 7-day embryos and for thigh and brain dissected from 11-day embryos. At 15 days, the presence of type M is definitely detectable in thigh muscle, while the appearance of type M in breast muscle and brain seems to be somewhat delayed. At 19 days, the relative quantities of types K and M in brain and skeletal muscle are still not the same as observed with adult tissues; the adult ratio of these

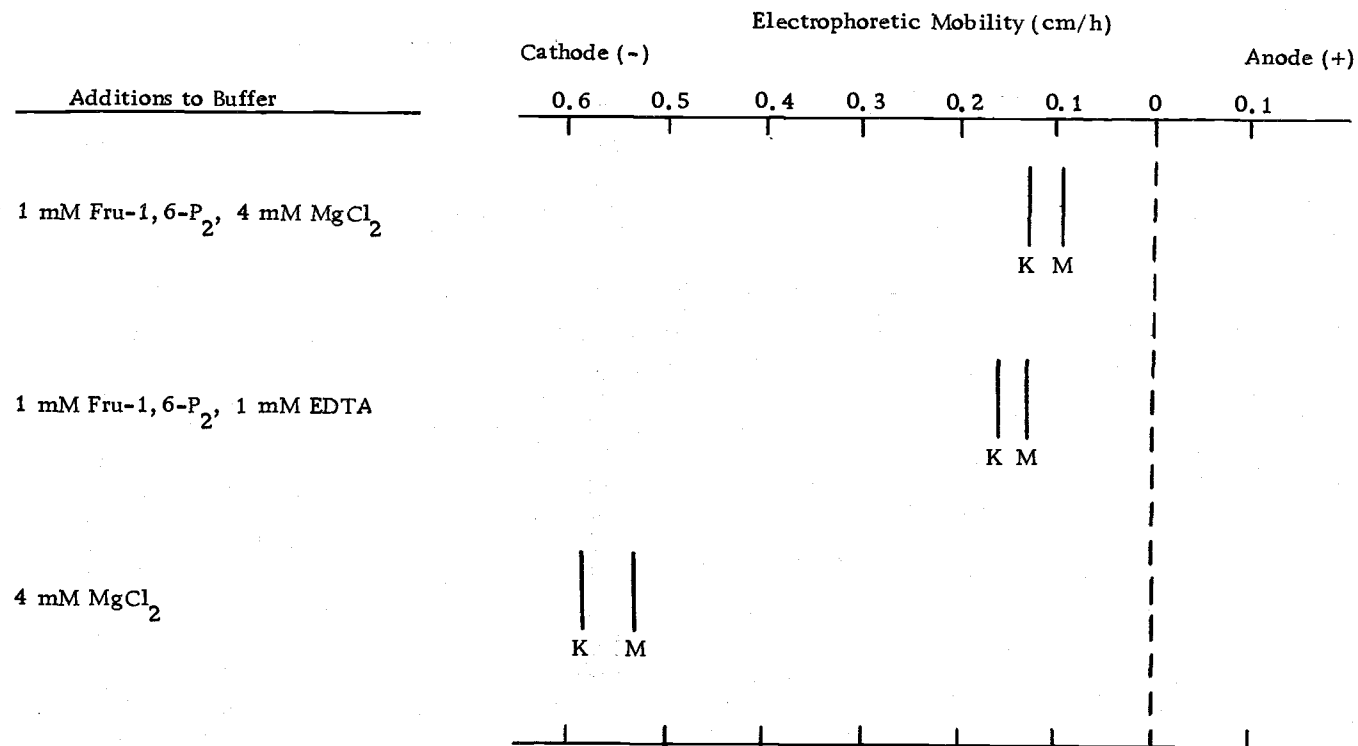


Fig. 11. Electrophoretic mobilities of pyruvate kinase isozymes of chicken in various buffers on cellulose acetate. The buffers consisted of 0.02 M Tris-HCl (pH 8.0 at 4°), 0.5 M sucrose, 10 mM  $\beta$ -mercaptoethanol, plus the additions indicated. Measurements were made for the type M isozyme of adult breast muscle (pectoralis major) and the type K of adult liver. The voltage was 250 volts.



isozymes is achieved after hatching, which occurs at 20-21 days.

Liver and kidney contain only one isozyme (type K), with no evidence for an anodic band such as the type L band seen in mammalian liver, kidney, intestines, and erythrocytes. To minimize the possibility that there were additional bands of pyruvate kinase in chicken liver that were not separated by our regular electrophoresis buffer, extracts of liver were electrophoresed in four other buffers (Fig. 10) and for varying periods of time. Only one band of pyruvate kinase was detected in liver under any of these conditions.

#### Electrophoretic Mobilities under Various Conditions

During the investigations of the isozyme patterns in various tissues, a number of different buffer systems were tried, making it possible to compare the electrophoretic mobilities of the individual isozymes under different conditions. The electrophoretic mobilities of chicken type K and type M isozymes, expressed in cm/h, are illustrated in Fig. 11 for three different buffers. The use of both fructose 1,6-diphosphate and  $\text{MgCl}_2$  gave optimal separation of type K and type M while keeping the isozymes relatively near the origin.

The mobilities of the isozymes in mouse tissues were investigated at the same time that the different buffers were tested on the chicken isozymes, and these results are shown in Fig. 12. The effect of fructose 1,6-diphosphate on the position of type M relative to the

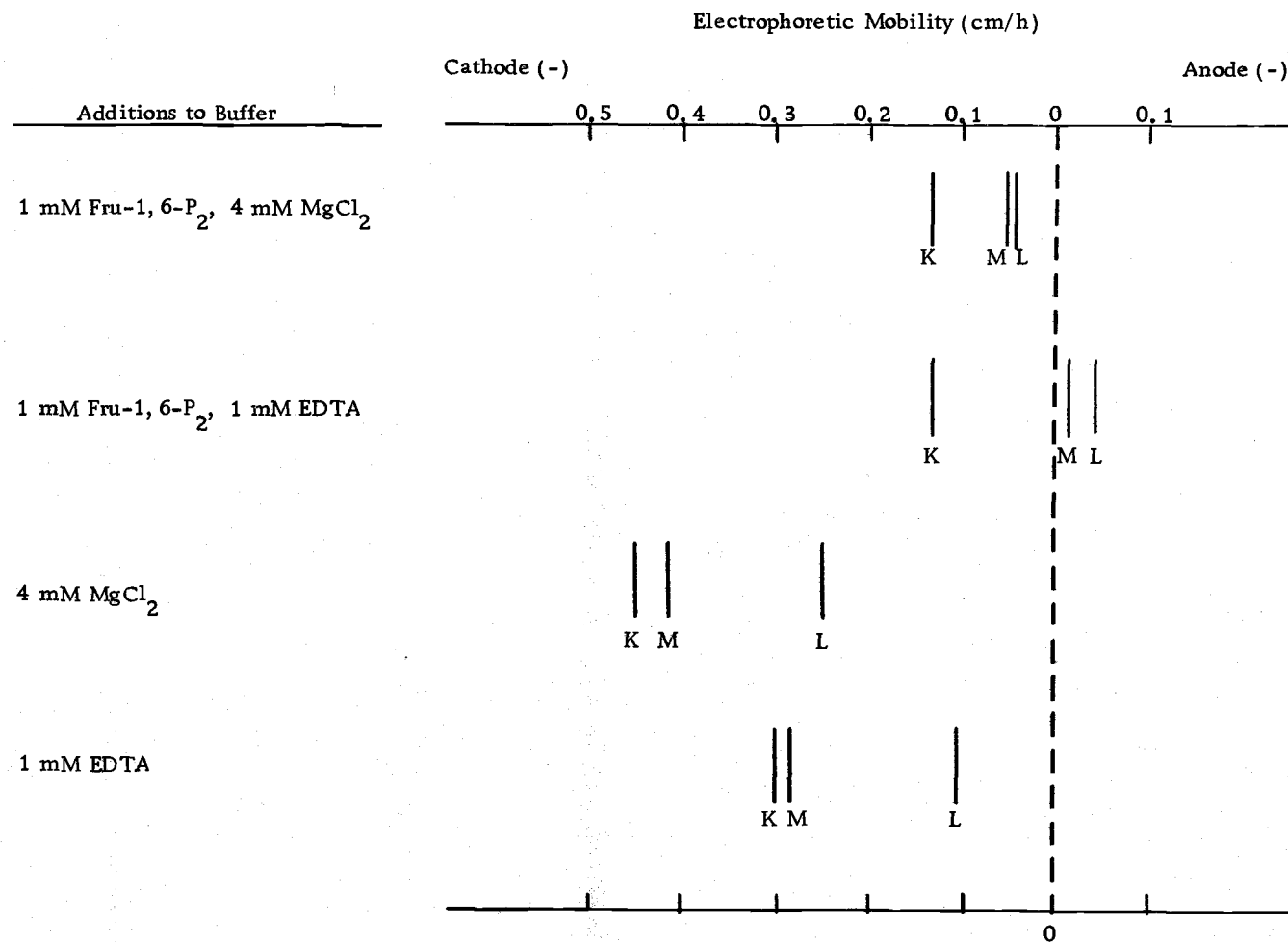


Fig. 12. Electrophoretic mobilities of the pyruvate kinase isozymes of mouse in various buffers on cellulose acetate. The buffers consisted of 0.02 M Tris-HCl (pH 8.0 at 4°), 0.5 M sucrose, 10 mM  $\beta$ -mercaptoethanol, plus the additions indicated. Measurements were made for the type L isozyme of adult liver, the type M isozyme of adult heart, and the type K isozyme of adult liver. The voltage was 250 volts.

other two isozymes is quite striking. In the presence of fructose 1,6-diphosphate type M migrates close to the position of type L, whereas in the absence of fructose 1,6-diphosphate type M runs close to the position of type K.

The results of several different buffers on the mobility of the bovine type L pyruvate kinase isozyme is illustrated in Fig. 13. In Fig. 14 are shown the results of two different concentrations of Fru-1,6- $P_2$  on the mobilities and separation of bovine type M and type K. Decreasing the fructose 1,6-diphosphate concentration has the effect of markedly reducing the separation of type K and type M. (Note the considerable difference in scales of electrophoretic mobilities in Figs. 13 and 14. Because of this difference and the fact that the same buffers were not used in all cases, it was impossible to show all three bovine isozymes in the same figure.)

#### Kinetic and Immunological Properties of Pyruvate Kinase from Chicken Tissues

Figs. 15 and 16 illustrate the dependence of chicken pyruvate kinase activity on P-enolpyruvate concentration, using extracts of adult chicken liver and fetal chick thigh muscle, respectively, both of which appear on electrophoresis to contain only type K pyruvate kinase. Both the adult liver and the fetal muscle pyruvate kinases exhibit sigmoidal kinetics without fructose 1,6-diphosphate, but

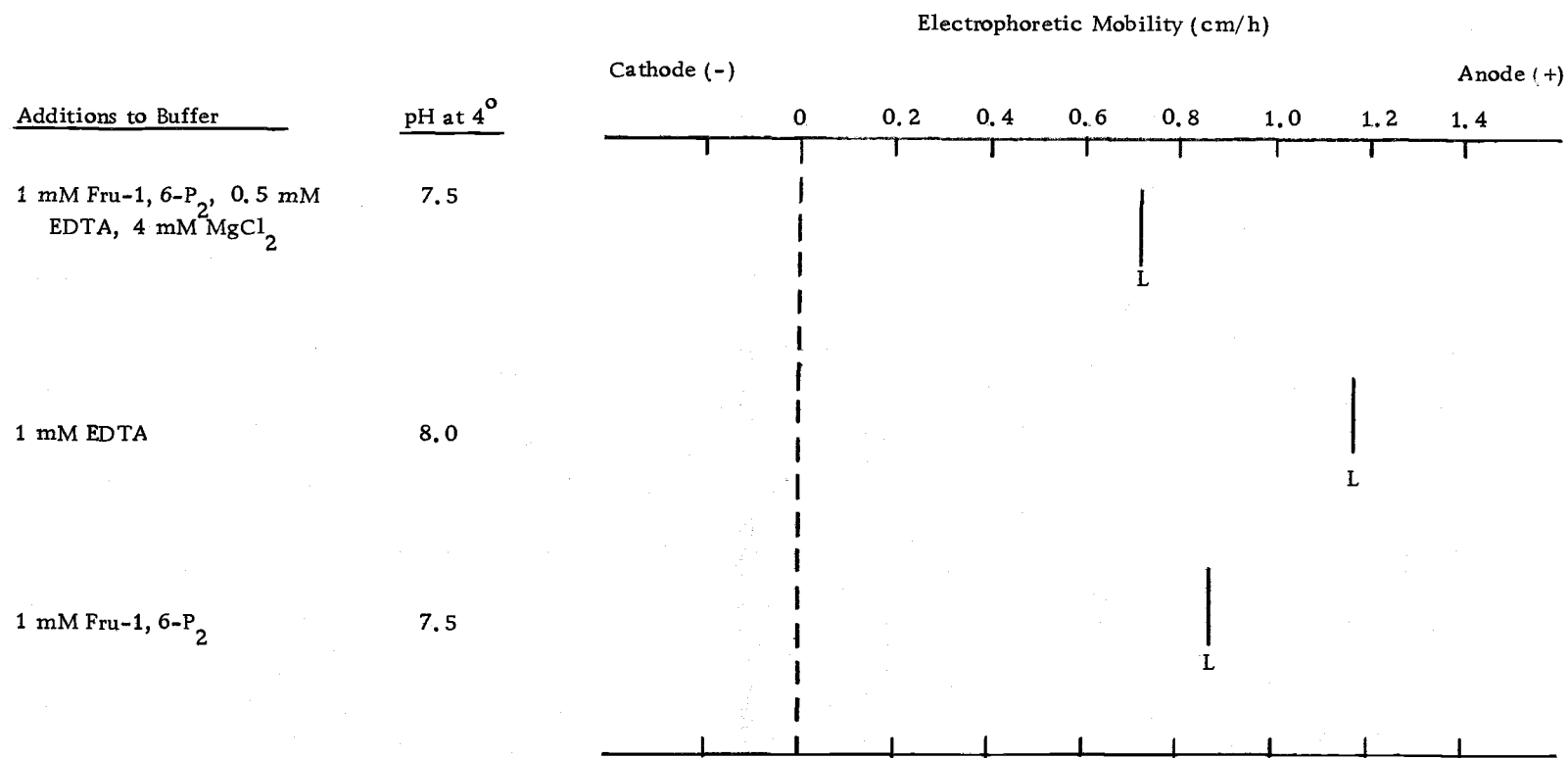


Fig. 13. Electrophoretic mobility of the type L isozyme of bovine pyruvate kinase in various buffers on cellulose acetate. The buffers consisted of 0.02 M Tris-HCl, 0.5 M sucrose, 10 mM  $\beta$ -mercaptoethanol, plus the additions indicated. The pH at 4° was either 7.5 or 8.0 as indicated. The measurements are based on the mobilities of both the liver and the erythrocyte isozymes.

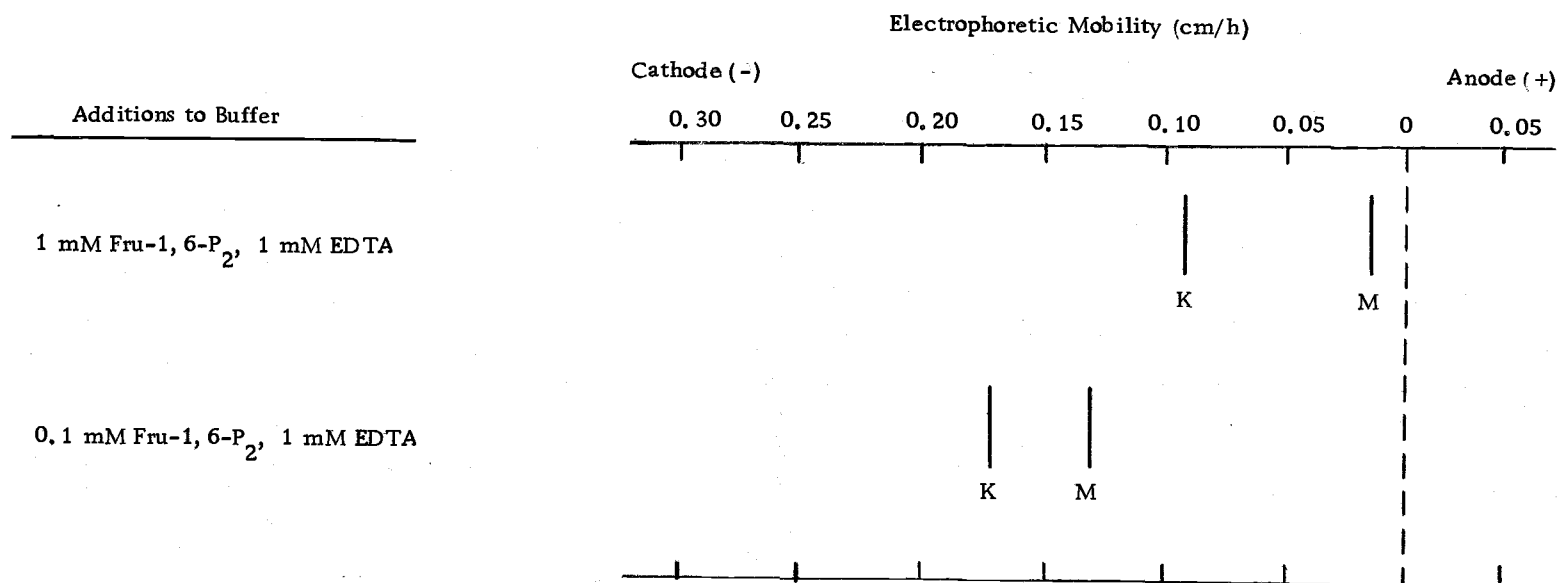


Fig. 14. Electrophoretic mobilities of the type K and type M pyruvate kinase isozymes of beef in two different concentrations of Fru-1, 6-P<sub>2</sub> on cellulose acetate. The buffers consisted of 0.02 M Tris-HCl (pH 8.0 at 4°), 0.5 M sucrose, 10 mM β-mercaptoethanol, and either 1.0 mM or 0.1 mM Fru-1, 6-P<sub>2</sub> as indicated. The voltage was 200 volts. Measurements were made on the K<sub>4</sub> and M<sub>4</sub> isozymes in tissue extracts of pregnant uterus.

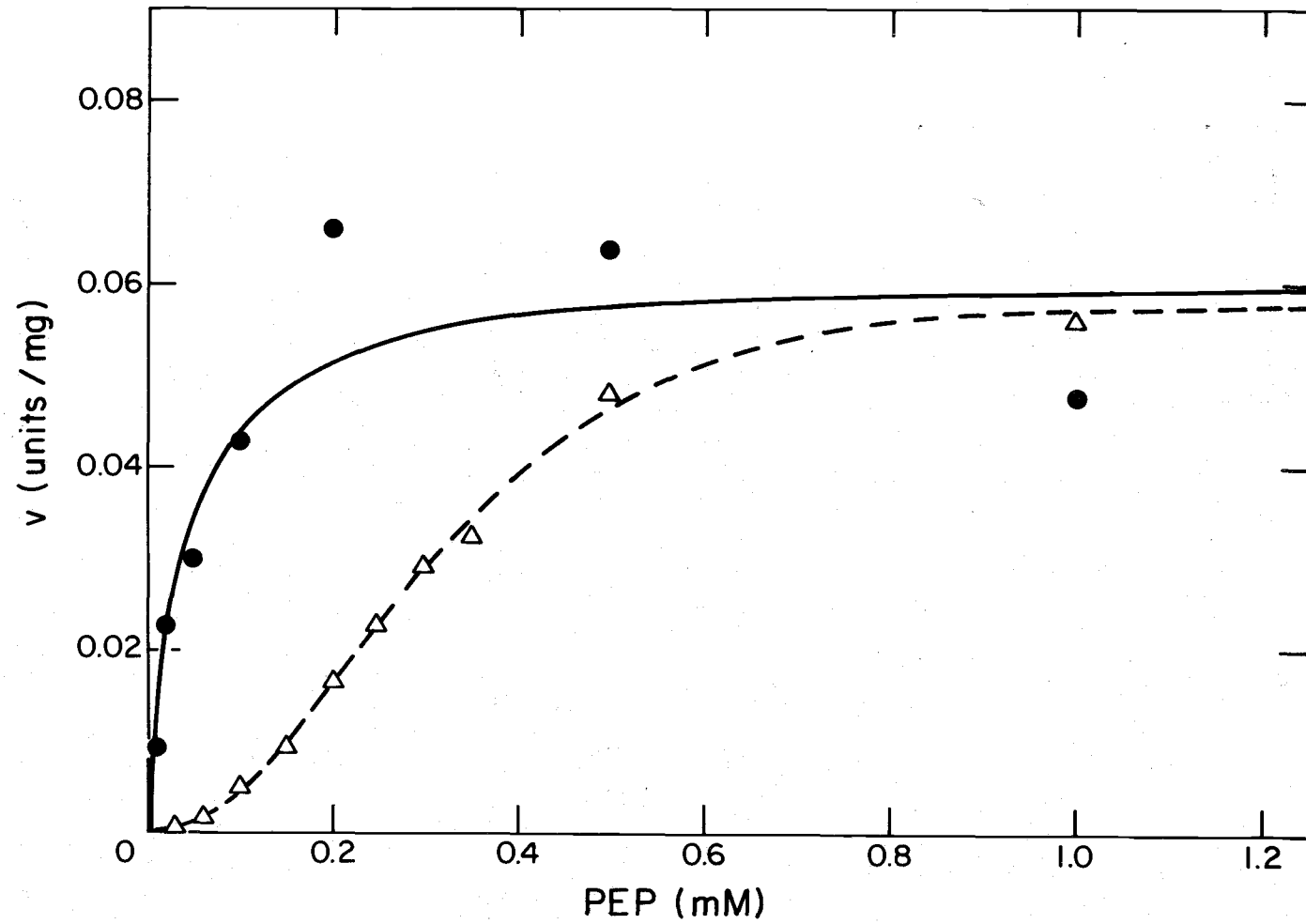


Fig. 15. Velocity of pyruvate kinase from adult chicken liver as a function of P-enolpyruvate (PEP) concentration in the presence (●) and absence (Δ) of 1.0 mM fructose 1,6-diphosphate. The concentration of ADP was 1.0 mM.

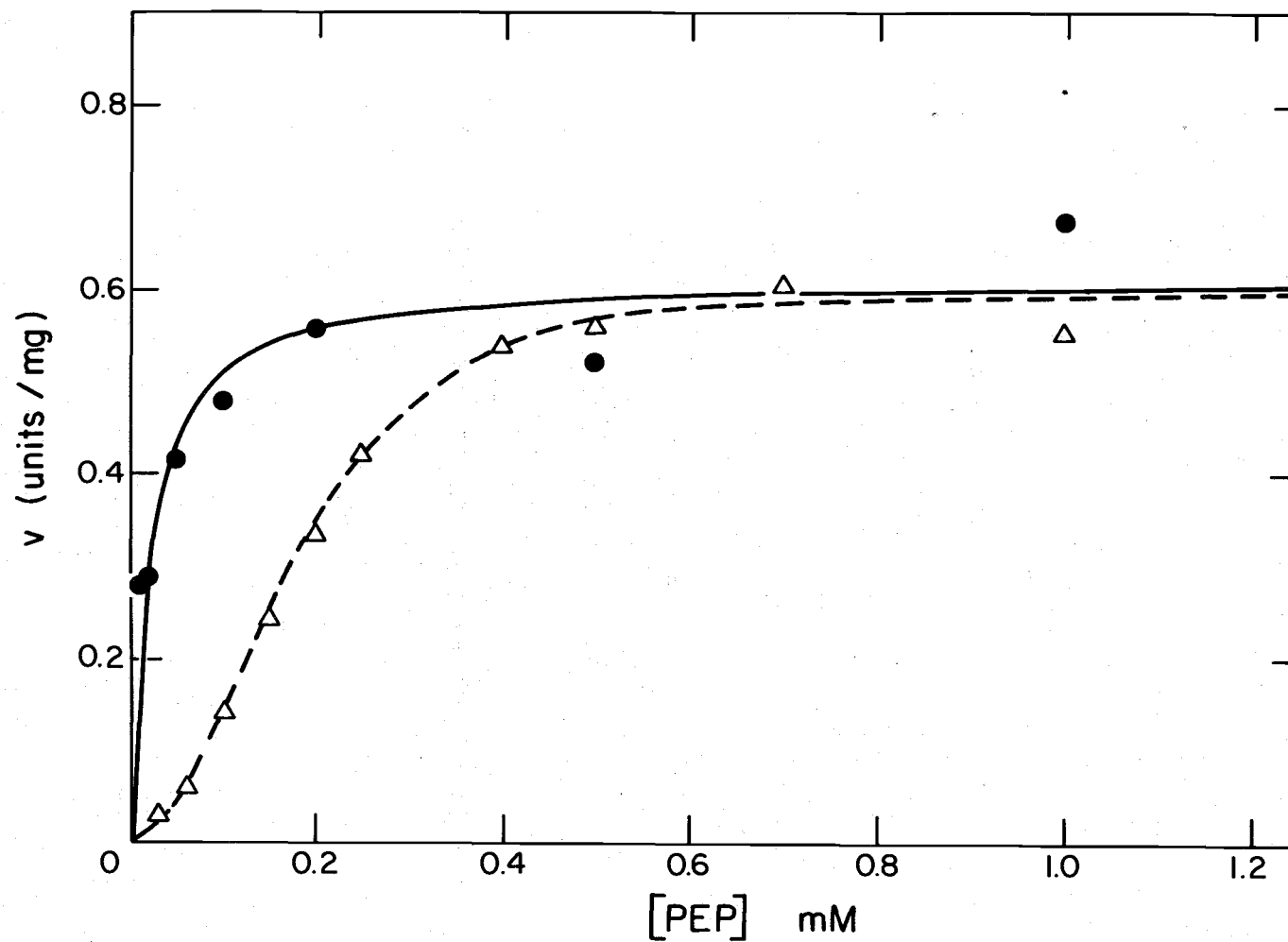


Fig. 16. Velocity of pyruvate kinase from thigh muscle of 11-day fetal chick as a function of P-enolpyruvate (PEP) concentration in the presence (●) and absence (Δ) of 1.0 mM fructose 1,6-diphosphate. The concentration of ADP was 1.0 mM.

Table VII. Kinetic constants for pyruvate kinase activity of chicken tissues.<sup>a</sup>

| Parameter   | Extract of<br>Adult Chicken<br>Liver | Extract of<br>Thigh Muscle from<br>11-day Fetal Chick | Purified Skeletal<br>Muscle<br>Isozyme |
|---|--------------------------------------|---|--|
| Hill coefficient  | 2.1                                  | 1.9   | 1.0                                    |
| $S_{0.5}$ for P-enolpyruvate (mM)   | 0.33                                 | 0.20  | 0.04                                   |
| $K_M$ for P-enolpyruvate in the<br>presence of Fru-1, 6-P <sub>2</sub> (mM) | 0.04                                 | 0.02  | 0.04                                   |

<sup>a</sup> Kinetics of adult liver and fetal thigh muscle extracts were studied at 1.0 mM ADP and pH 7.5. Parameters of the muscle isozyme are from Cardenas et al. (1975b) and were determined at 1.32 mM ADP and pH 7.0.



hyperbolic curves in the presence of fructose 1, 6-diphosphate.

Pyruvate kinase from adult breast muscle (which shows only type M on electrophoresis), on the other hand, has hyperbolic kinetics that are unaffected by fructose 1, 6-diphosphate (Cardenas et al., 1975b). The Michaelis constants, values for  $S_{0.5}$ , and Hill coefficients obtained from the kinetic studies are listed in Table VII. The Michaelis constants and conclusions regarding fructose 1, 6-diphosphate activation reported here are in approximate agreement with earlier studies on the pyruvate kinase in extracts of chicken liver and muscle reported by Leveille (1969) and with the data on pigeon liver pyruvate kinase reported by Gabrielli and Baldi (1972, 1973).

The specific activity (micromoles per min per mg protein) of enolase in extracts of 11-day fetal thigh muscle was small (0.08) compared to the specific activity of pyruvate kinase (0.7). However, in extracts of adult chicken liver, the specific activity of enolase was slightly greater (0.08) than that of pyruvate kinase (0.06). Kinetic analysis of enolase activity in chicken liver gave a  $K_m$  for P-enolpyruvate of 0.3 mM. These high quantities of enolase in the liver extracts would complicate the assays for pyruvate kinase by lowering the concentration of P-enolpyruvate. However, the equilibrium between P-enolpyruvate and 2-phosphoglycerate catalyzed by enolase

lies toward P-enolpyruvate in a ratio of about 4.3 to 1 (Wold and Ballou, 1957), so that the concentration of P-enolpyruvate could be decreased due to the action of enolase only by 20%, even if equilibrium for the enolase reaction were reached during the time of the pyruvate kinase assays. For most of the assays, particularly at the higher P-enolpyruvate concentrations, the enolase reaction would not reach equilibrium, and the error in the P-enolpyruvate concentration would be 10% or less. Correcting the P-enolpyruvate concentration for this effect did not appear to cause significant changes in the curves, so this correction has not been included in the data presented here.

Fig. 17 shows the dependence of adult liver pyruvate kinase activity on ADP concentration at 0.2 mM P-enolpyruvate. No appreciable substrate inhibition is observed at the ADP levels used (up to 5 mM).

Rabbit antibodies produced against bovine skeletal muscle pyruvate kinase neutralize chicken skeletal muscle pyruvate kinase (Cardenas et al., 1975b). In Fig. 18 it is shown that this antiserum also neutralizes virtually all of the pyruvate kinase in fetal thigh and adult liver of chicken, which appear in zymograms to contain only type K. Also, antiserum prepared against purified chicken type M pyruvate kinase is effective in neutralizing the activity in adult chicken liver. Thus, the chicken type K and type M pyruvate kinases are immunologically similar, consistent with the report of Imamura

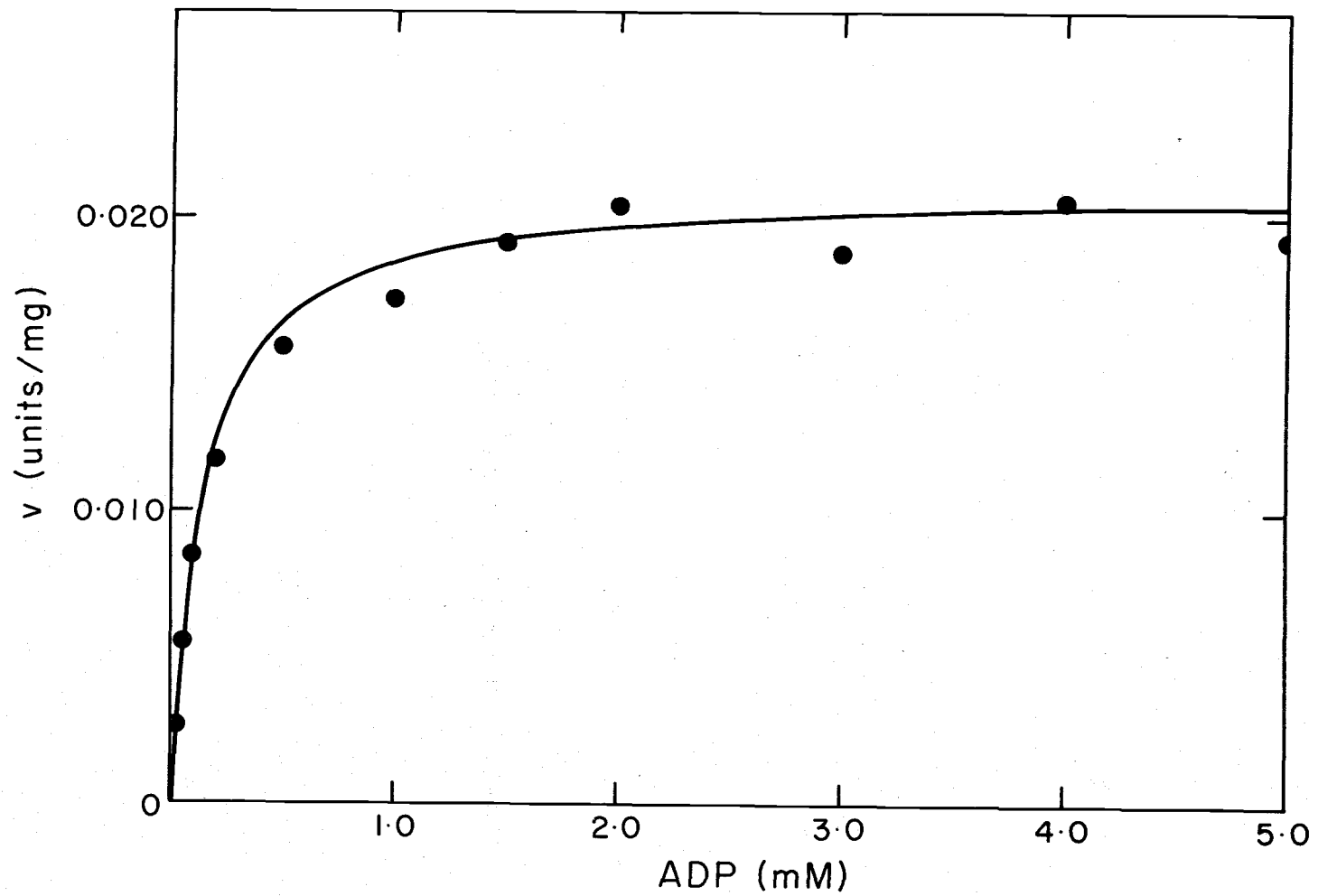


Fig. 17. Velocity of pyruvate kinase from adult chicken liver as a function of ADP concentration. The P-enolpyruvate concentration was 0.2 mM.

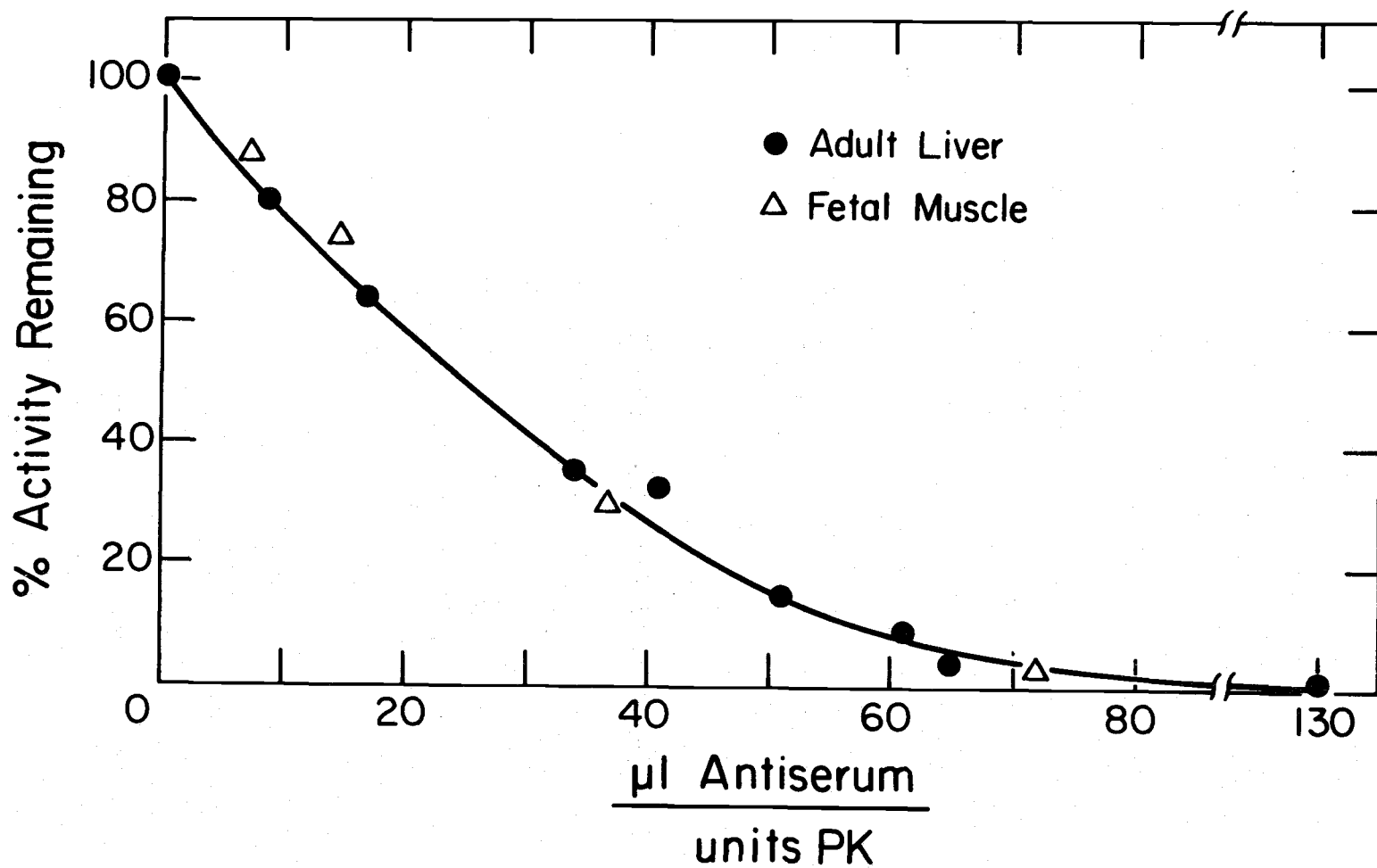


Fig. 18. Pyruvate kinase activity remaining in adult chicken liver (●) and fetal chick thigh muscle (Δ) extracts after treatment with rabbit antiserum prepared by inoculation with bovine pyruvate kinase type M. The methods employed are described in the Materials and Methods section.

et al. (1972) that mammalian type K and M pyruvate kinases are immunologically cross-reactive but distinct from the mammalian type L.

#### Partial Purification of Bovine Type K<sub>4</sub> Pyruvate Kinase

A typical preparation of bovine kidney pyruvate kinase is summarized in Table VIII. Heat treatment and ammonium sulfate fractionation yield a preparation with a specific activity of 5-7 micromoles/min/mg. This preparation contains both K-L and K-M hybrids, the electrophoretic pattern appearing to resemble closely the original extract of kidney despite the fact that type L<sub>4</sub> pyruvate kinase is precipitated from liver extracts at lower ammonium sulfate concentrations (Cardenas and Dyson, 1973).

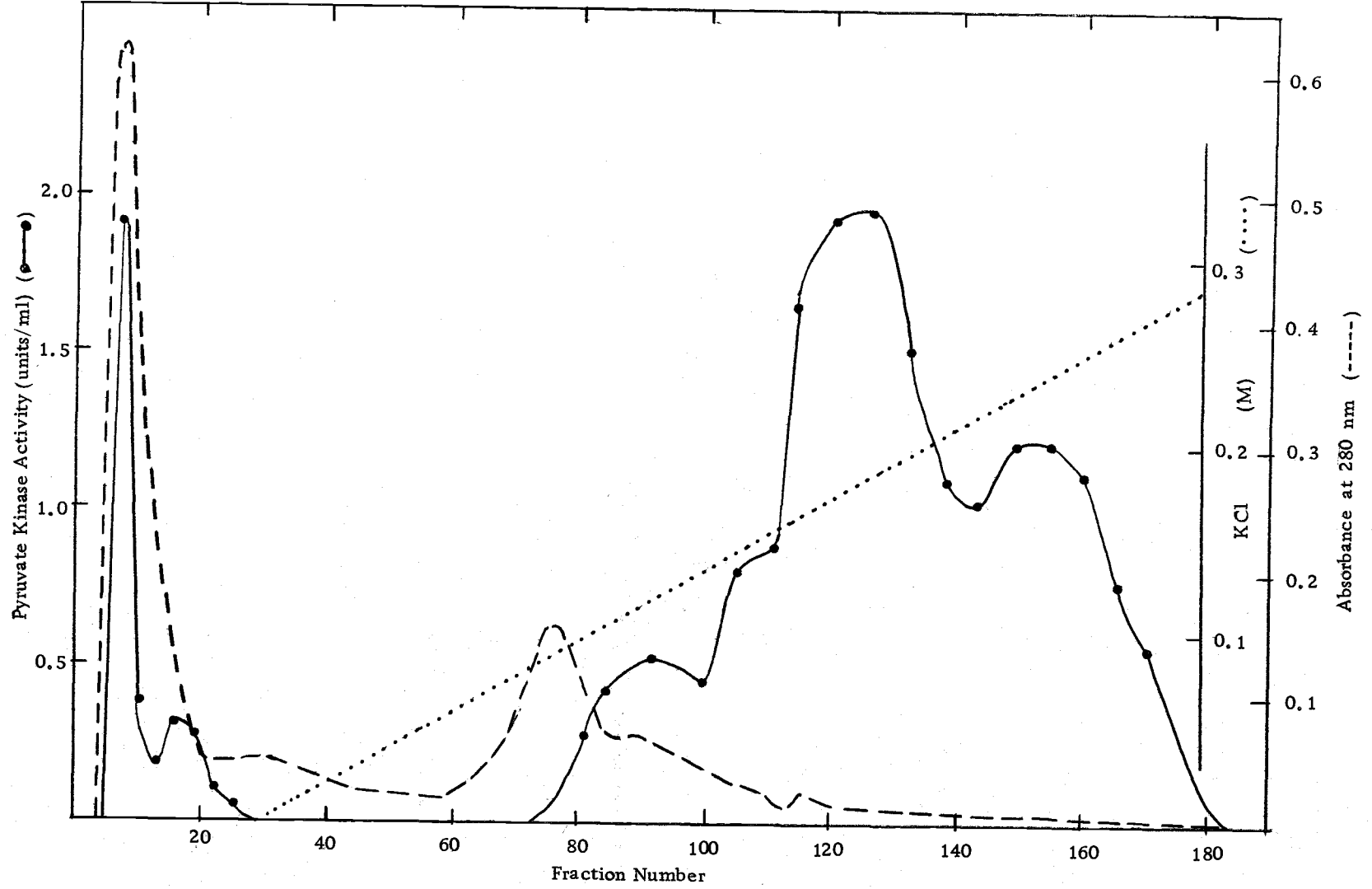
The elution patterns of the bovine kidney pyruvate kinase preparations from CM-Sephadex C-50 columns are illustrated in Figs. 19 and 20. A peak of pyruvate kinase activity, usually followed by a smaller shoulder, is eluted in the buffer wash of the column along with a considerable quantity of other proteins. This activity consists of the L<sub>4</sub> isozyme and some (or perhaps all) of the K-L hybrids.

At pH 6.5 (Fig. 19) the KCl gradient elutes the K-M hybrids and K<sub>4</sub> pyruvate kinase as a close series of activity peaks, with the K<sub>4</sub> coming last, separated to some degree from K<sub>3</sub>M. At higher pH,

Table VIII. Partial co-purification of bovine kidney types  $K_3M$  and  $K_4$  pyruvate kinases.

| Fraction   | Volume    | Total Pyruvate        | Total Protein | Specific Activity        | Yield    |
|--|-----------|-----------------------|---------------|--------------------------|----------|
|  |           | Kinase Activity       |               |                          |          |
|  | <u>ml</u> | <u>micromoles/min</u> | <u>mg</u>     | <u>micromoles/min/mg</u> | <u>%</u> |
| 1. Extract from 136 g of bovine kidney   | 300       | 5,300                 | 10,650        | 0.50                     | 100      |
| 2. Heat step   | 235       | 4,610                 | 2,350         | 1.96                     | 87       |
| 3. Ammonium sulfate fractionation  | 9.0       | 3,430                 | 538           | 6.4                      | 68       |
| 4. CM-Sephadex C-50 chromatography<br>at pH 7.7 (yield of K <sub>3</sub> M-K <sub>4</sub> mixture) |           | 1,310                 | 39.1          | 33                       | 25       |

Fig. 19. CM-Sephadex C-50 chromatography of bovine kidney pyruvate kinase isozymes at pH 6.5. 270 mg of protein with a specific activity of 5.8 were loaded on a 2.5 x 40 cm column and eluted at a flow rate of 30 ml/h into 6.0 ml fractions. A gradient of 0 to 0.30 M KCl in a total volume of 1.0 liter was applied as indicated. The last peak of activity eluted by the gradient consisted of type K<sub>4</sub> pyruvate kinase largely free of contaminating K<sub>3</sub>M, which was eluted in the next to last peak. For details of the procedure, see the Materials and Methods section. For this column only the assay medium contained 1.0 mM rather than 6.0 mM P-enolpyruvate.





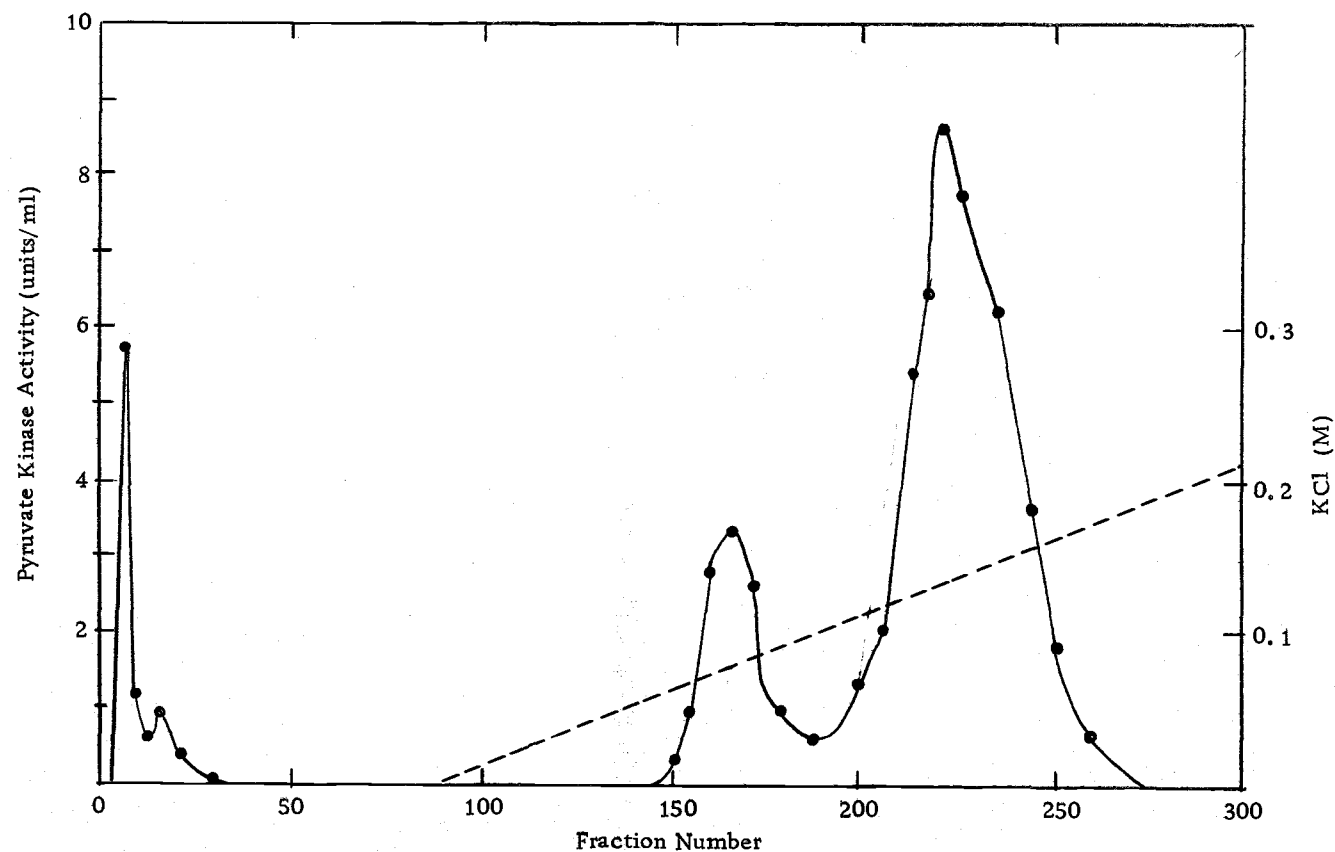


Fig. 20. CM-Sephadex C-50 chromatography of bovine kidney pyruvate kinase isozymes at pH 7.7. 538 mg of protein with a specific activity of 6.4 were loaded on a 2.5 x 40 cm column and eluted at a flow rate of 30 ml/h into 5.0 ml fractions. A gradient of 0 to 0.20 M KCl in a total volume of 1.0 liter was applied as indicated. The last peak of activity eluted by the gradient was a mixture of  $K_3^M$  and  $K_4$  pyruvate kinases. For details of the procedure, see the Materials and Methods section.

illustrated by the pH 7.7 column in Fig. 20, the K-M hybrids and  $K_4$  are eluted by the gradient as two peaks, the first peak consisting of a mixture of  $M_4$ ,  $KM_3$ , and  $K_2M_2$ , and the second peak consisting of a fairly homogeneous distribution of  $K_3M$  and  $K_4$ . The reason for the separation of these isozymes into two separate classes during elution at the higher pH values is unknown. The yield of  $K_3M$ - $K_4$  mixture from the pH 7.7 column is indicated in Table VIII.

Type  $K_4$  pyruvate kinase could be obtained substantially free of  $K_3M$  only from CM-Sephadex columns run at pH 6.5. Generally only the fractions near the end of the activity peak had pure  $K_4$ . The  $K_4$  preparations had specific activities of 20 or more units per mg protein.

#### Properties of Bovine Type $K_4$ Pyruvate Kinase

The velocity of the partially purified kidney type  $K_4$  pyruvate kinase with respect to P-enolpyruvate concentration is shown in Fig. 21. The velocity profile is slightly sigmoidal, with a Hill coefficient of approximately 1.5 and a  $S_{0.5}$  of about 1.1 mM (Table IX). The addition of fructose 1,6-diphosphate seems to have a slight activating effect, giving a profile with a Hill coefficient of 1.0 and apparently a somewhat higher maximal velocity.

In an attempt to check for possible alteration of the kinetic properties of type  $K_4$  pyruvate kinase during preparation, a velocity profile of the pyruvate kinase in a fresh extract of an embryo was

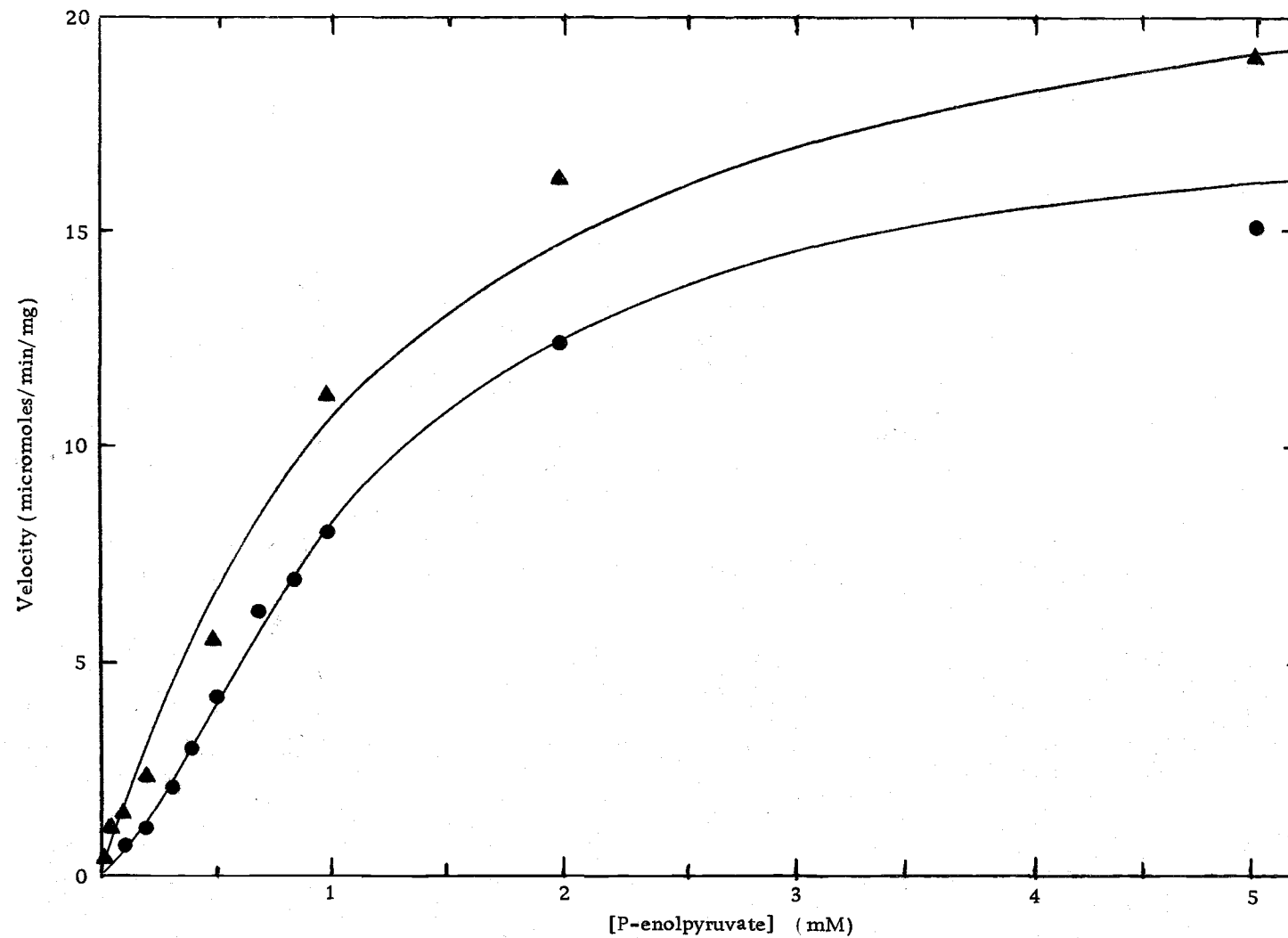


Fig. 21. Velocity of partially purified bovine kidney type  $K_4$  pyruvate kinase as a function of P-enolpyruvate concentration in the presence (▲) and absence (●) of 1.0 mM fructose 1, 6-diphosphate.

obtained (Fig. 22). The embryo was chosen as a source of  $K_4$  since it contains mostly this isozyme and only small quantities of  $K_3M$ ,  $K_2M_2$ , and  $M_4$ , in contrast to other bovine tissues that have greater quantities of hybrids. The kinetic profile obtained for the embryo pyruvate kinase resembles the profile of the kidney type  $K_4$  pyruvate kinase in that it is only slightly sigmoidal and the activation by fructose 1,6-diphosphate is not great. However, the  $S_{0.5}$  for P-enolpyruvate is substantially less than that of the kidney  $K_4$  both in the presence and absence of fructose 1,6-diphosphate. The kinetic parameters for both the kidney  $K_4$  and embryo preparations are listed in Table IX.

Bovine kidney type  $K_4$  pyruvate kinase, like type  $K_4$  from other mammalian sources, is strongly inhibited by low concentrations of L-phenylalanine or L-alanine. The effects of these amino acids on the activity at 0.5 mM P-enolpyruvate concentration are shown in Figs. 23 and 24, and the concentrations of these amino acids required to produce 50% inhibition (designated as  $K_i$ ) are listed in Table IX. Both L-phenylalanine and L-alanine strongly inhibit bovine type  $K_4$  pyruvate kinase. The addition of fructose 1,6-diphosphate fully reverses this inhibition at the lower amino acid concentrations and partially reverses the inhibition even at 5 mM concentrations of the amino acids.

An immunological titration of the partially purified type  $K_4$

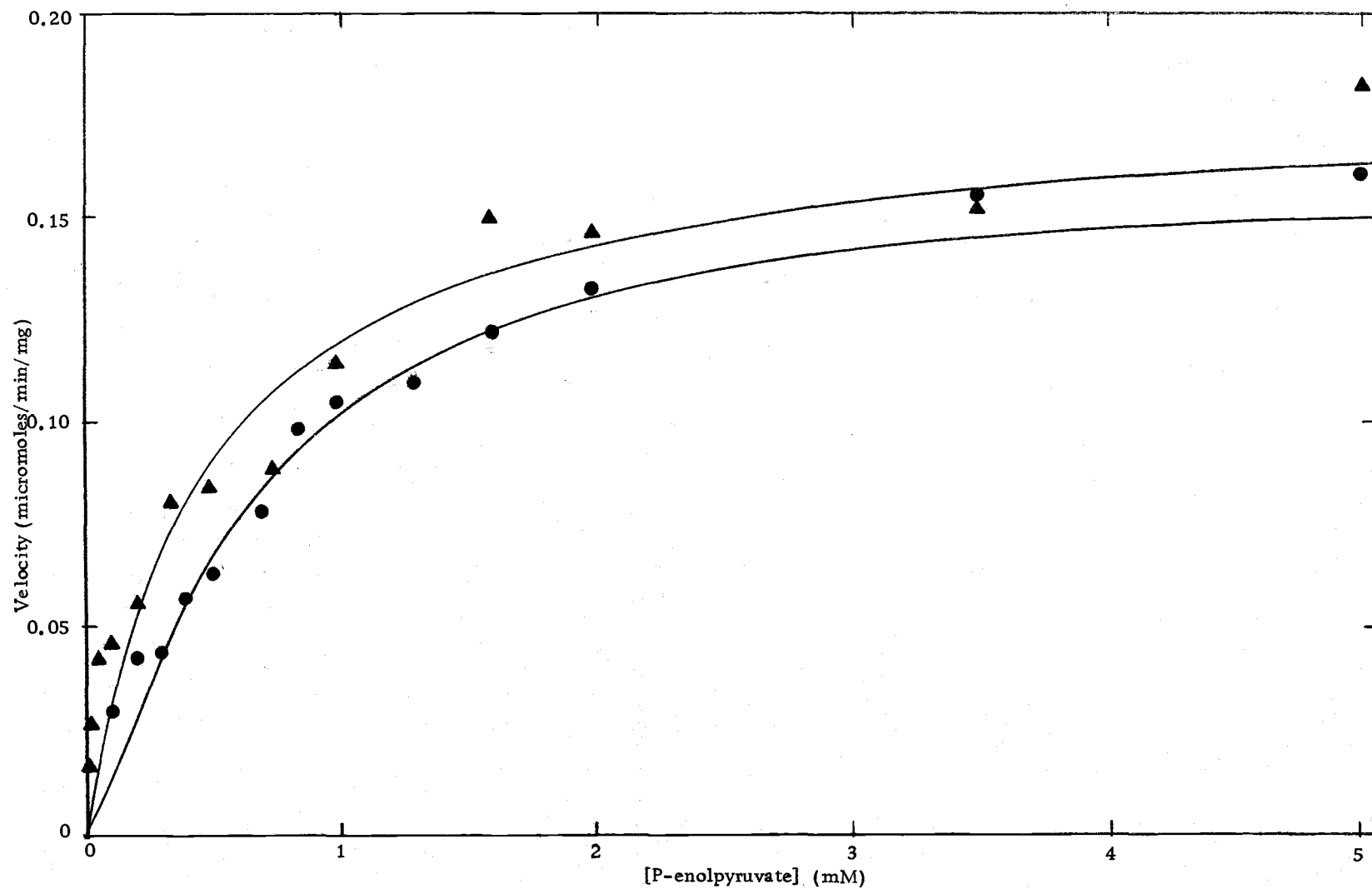


Fig. 22. Velocity of pyruvate kinase in extract of bovine embryo as a function of P-enolpyruvate concentration in the presence (▲) and absence (●) of 1.0 mM fructose 1, 6-diphosphate. The embryo was taken at approximately 1 1/2-2 months gestation, and the extract contained small amounts of  $K_3M$ ,  $K_2M_2$ , and  $M_4$  in addition to the predominant  $K_4$  isozyme (see electrophoresis in Fig. 5).

Table IX. Kinetic parameters of bovine type  $K_4$  pyruvate kinase.

| Parameter   | Partially<br>Purified<br>Kidney<br>Type $K_4$ | Extract<br>of<br>1 1/2 mo.<br>Embryo |
|---|---|--------------------------------------|
| $S_{0.5}$ for P-enolpyruvate (mM)   | 1.1   | 0.66                                 |
| Hill coefficient ( $n_H$ )  | 1.5   | 1.3                                  |
| $K_m$ for P-enolpyruvate in the<br>presence of 1.0 mM Fru-1, 6-P <sub>2</sub> | 1.3   | 0.51                                 |
| $n_H$ in the presence of<br>1.0 mM Fru-1, 6-P <sub>2</sub>                    | 1.0   | 1.0                                  |
| $K_i$ for Phe at 0.5 mM<br>P-enolpyruvate (mM)                                | 0.11  |                                      |
| $K_i$ for Ala at 0.5 mM<br>P-enolpyruvate (mM)                                | 0.042   |                                      |

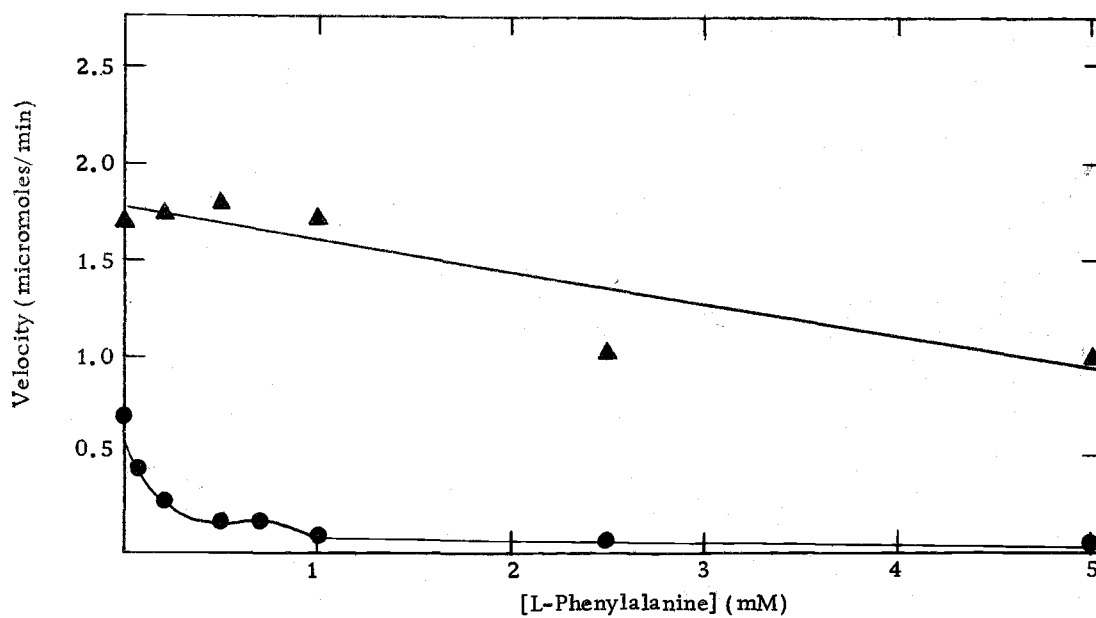


Fig. 23. The effect of L-phenylalanine on the velocity of partially purified bovine kidney type  $K_4$  pyruvate kinase at 0.5 mM P-enolpyruvate in the presence of (▲) and absence (●) of 1.0 mM fructose 1,6-diphosphate.

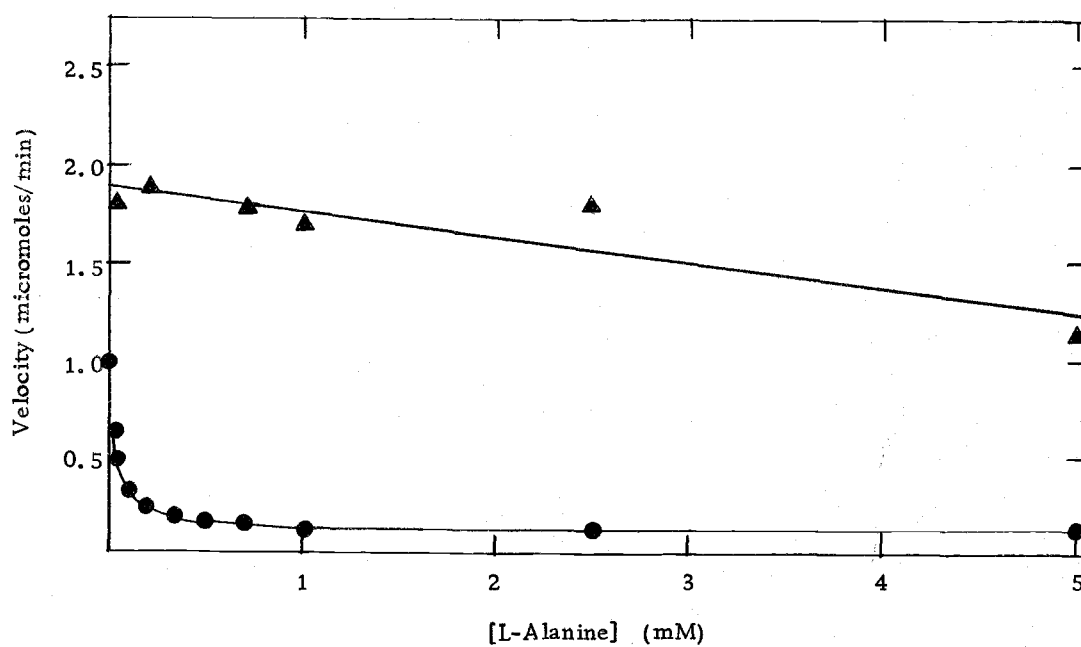


Fig. 24. The effect of L-alanine on the velocity of partially purified bovine kidney type  $K_4$  pyruvate kinase at 0.5 mM P-enolpyruvate in the presence (▲) and absence (●) of 1.0 mM fructose 1,6-diphosphate.



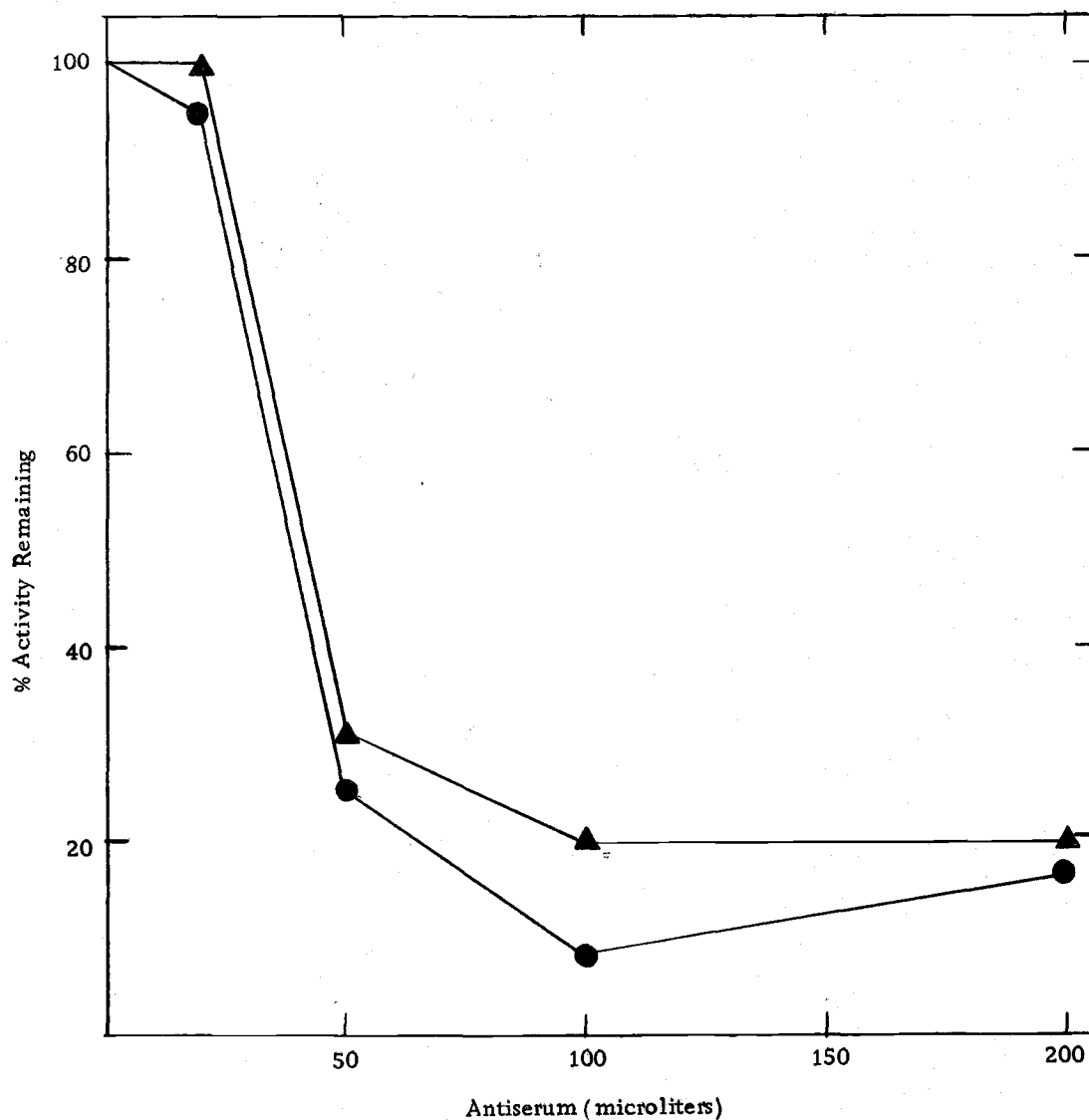


Fig. 25. Activity remaining in preparations of type  $M_4$  (●) and type  $K_4$  (▲) pyruvate kinase after treatment with chicken antiserum prepared by inoculation with bovine pyruvate kinase type  $M_4$ . The methods employed are described in the Materials and Methods section. The activities of type  $M_4$  and type  $K_4$  initially present were approximately equal.

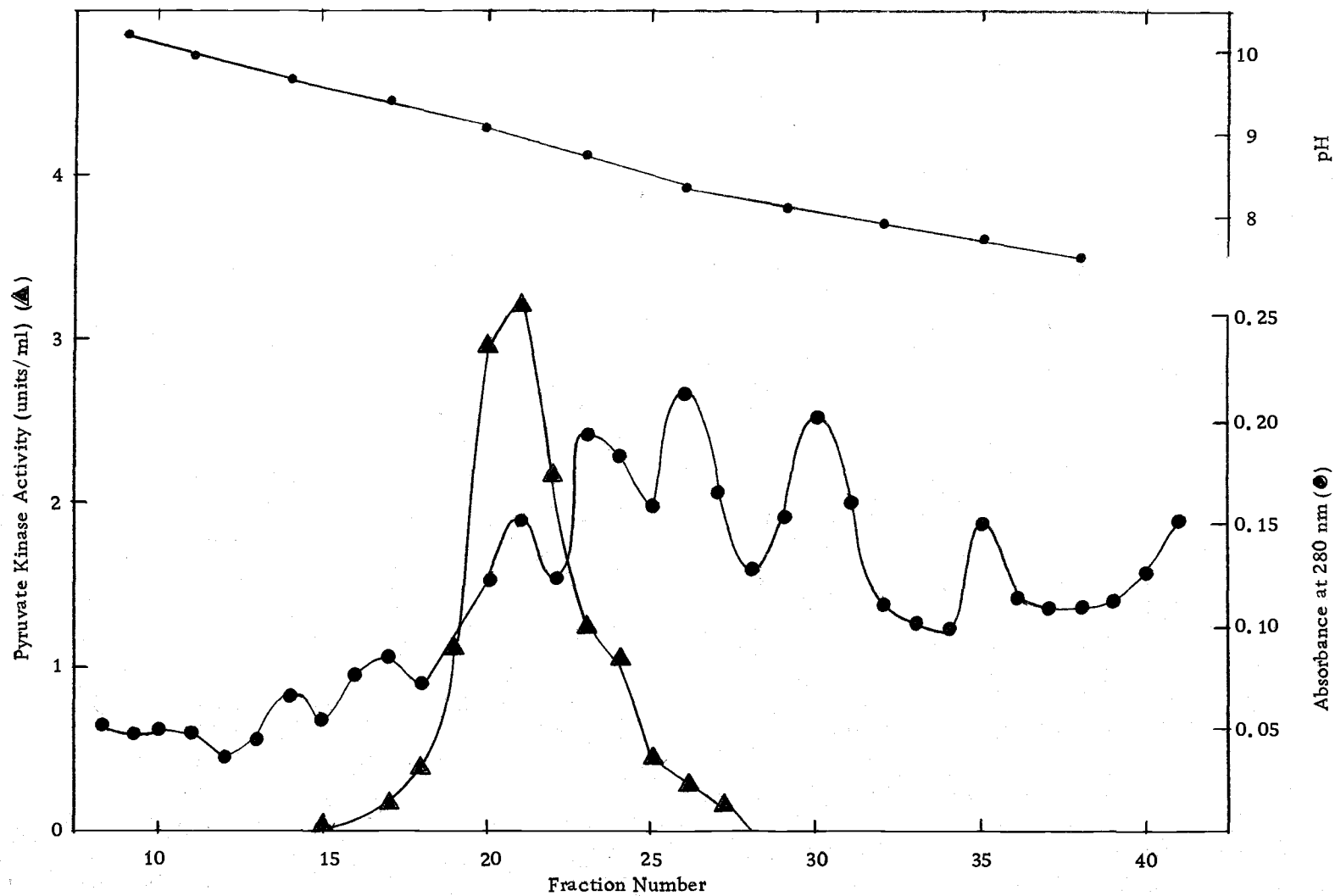


Fig. 26. Isoelectric focusing of partially purified bovine kidney type  $K_4$  pyruvate kinase. Seven mg of protein with a specific activity of 34 micromoles/min/mg were focused in LKB ampholines of pH range 7 to 10. The isoelectric pH was 9.0

with antiserum prepared against bovine type  $M_4$  pyruvate kinase is shown in Fig. 25. Type  $K_4$  is precipitated by anti- $M_4$ , confirming that the bovine  $K_4$  and  $M_4$  isozymes are immunologically similar, as is true for the corresponding rat isozymes (Imamura et al., 1972).

The result of isoelectric focusing of the partially purified type  $K_4$  pyruvate kinase in ampholines of pH range 7 to 10 is shown in Fig. 26. The isoelectric pH of type  $K_4$  is 9.0, which is very close to the value of 8.9 which I obtained for purified type  $M_4$  pyruvate kinase (Fig. 27).

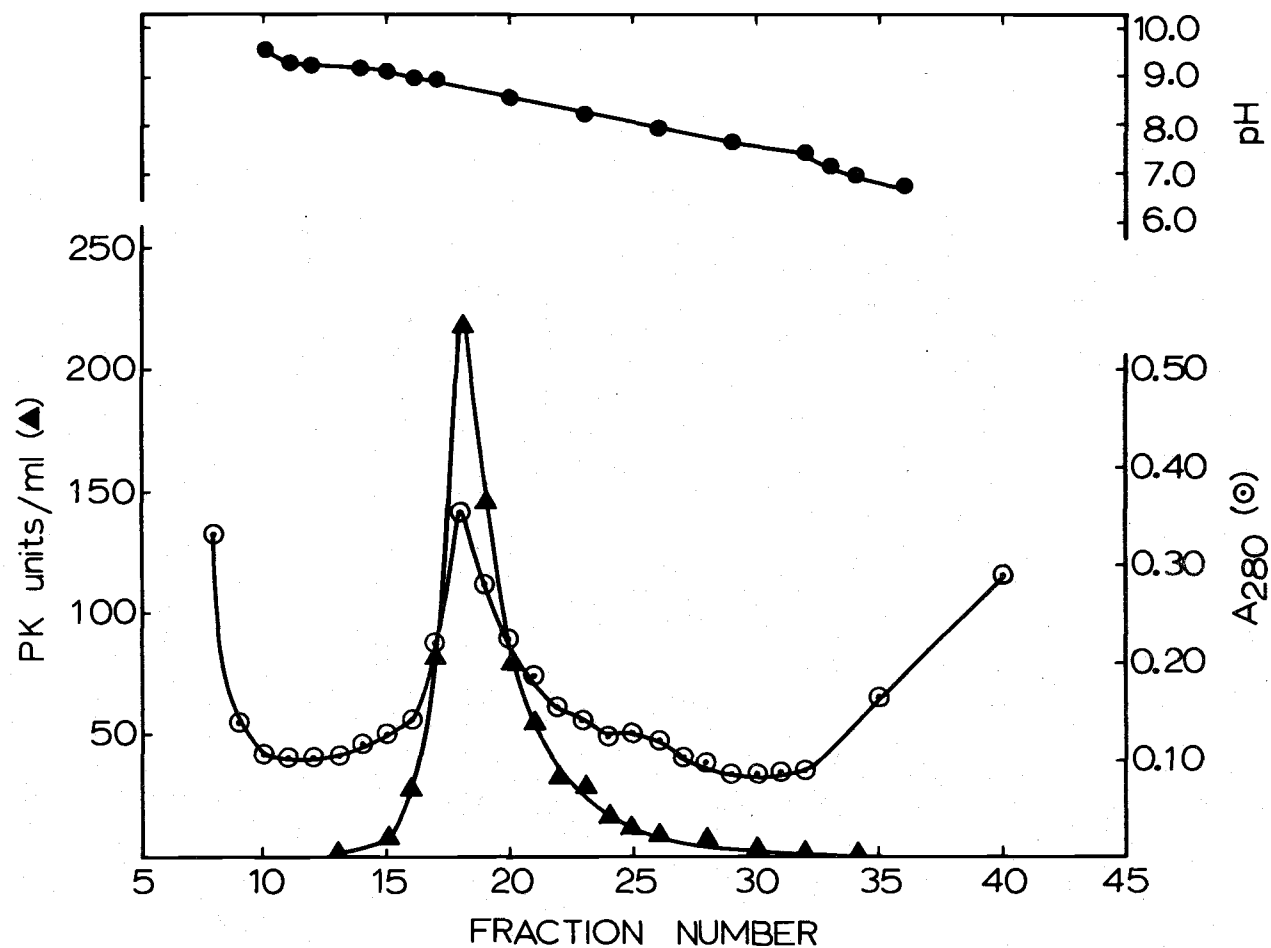


Fig. 27. Isoelectric focusing of bovine skeletal muscle pyruvate kinase (type  $M_4$ ). Six mg of protein with a specific activity of 161 micromoles/min/mg were focused in LKB ampholines of pH range 7 to 10. The isoelectric pH was 8.9. The slight asymmetry observed in this experiment was not observed in others and is probably due to a failure to reach equilibrium.

## DISCUSSION

Equilibrium Binding Studies

The values for average binding of P-enolpyruvate to bovine skeletal muscle pyruvate kinase listed in Table VI range from around two up to four moles of P-enolpyruvate bound per mole of pyruvate kinase. Many of the values lies between three and four. The numbers obtained are consistent with the hypothesis that a maximum of four P-enolpyruvate molecules bind to each tetramer of pyruvate kinase, or one P-enolpyruvate per subunit. The data are definitiely not consistent with the hypothesis that only two P-enolpyruvate molecules bind per tetramer of pyruvate kinase (Steinmetz and Deal, 1966).

The primary difficulty involved in obtaining a proper elution pattern from the gel filtration columns was the slow hydrolysis of P-enolpyruvate to pyruvate by pyruvate kinase even in the absence of ADP. Substitution of EDTA for  $MgCl_2$  substantially reduced the rate of this hydrolysis, and operation of the column at  $4^{\circ}$  further reduced it, enabling the expected elution patterns to be obtained. The hydrolysis was not entirely eliminated, however, and its greatest effect would have occurred in the ultracentrifuge method, where the enzyme solution was in contact with a fixed population of P-enolpyruvate molecules for a longer time.

From the work reported here on the P-enolpyruvate phosphatase

activity of the pyruvate kinase preparations, it was not possible to judge whether the activity was due to pyruvate kinase itself or to catalysis by trace amounts of an impurity, such as a nonspecific phosphatase. The fact that commercial preparations of rabbit skeletal muscle pyruvate kinase also hydrolyzed P-enolpyruvate in the absence of ADP made the former suggestion the more likely. Since this work was done, Erhard and Davis (1975) have reported the conclusion, based on observations under a number of different conditions, that the P-enolpyruvate phosphatase activity of rabbit skeletal muscle pyruvate kinase preparations is indeed due to pyruvate kinase itself.

Loss of P-enolpyruvate during the binding experiments can only result in erroneously low values for the number of binding sites for P-enolpyruvate. It may well be that this effect may account for the controversy in the past concerning the number of binding sites on pyruvate kinase.

These studies indicate that P-enolpyruvate is bound in the absence of  $Mg^{2+}$ . Although the removal of  $Mg^{2+}$  may have some effect on the affinity of the enzyme for P-enolpyruvate, it does not seem to be great if present at all, and the general conclusion regarding the number of binding sites is not affected.

Since these equilibrium binding studies reported here were done, several additional reports lending support to the concept of functional identity of all four subunits of pyruvate kinase have appeared. Both

the paper by Cardenas and Dyson (1973) and the paper by Kutzbach et al. (1973) reported Hill coefficients for P-enolpyruvate in excess of 2.0 for the purified liver ( $L_4$ ) isozyme. Since theoretically the Hill coefficient should not exceed the number of binding sites involved, these results indicate that more than two binding sites for P-enolpyruvate should be present. There have been reports indicating the presence of four sites for molecules binding to presumed allosteric sites on pyruvate kinase. Kayne and Price (1973) obtained Scatchard plots for binding of L-phenylalanine and L-alanine by equilibrium dialysis with rabbit skeletal muscle pyruvate kinase. These plots extrapolated to a maximum binding number of four of each amino acid per enzyme molecule. Competition experiments suggested that phenylalanine and alanine compete for the same site. Kutzbach et al. (1973) performed binding studies of fructose 1,6-diphosphate to purified pig liver pyruvate kinase and obtained extrapolated Scatchard plot values indicating four binding sites for this effector also.

Taking all the evidence together--the binding studies reported here, the reports mentioned in the introduction as indicating four active sites (Cottam and Mildvan, 1971; Hollenberg et al., 1971; Kayne, 1971), and the reports indicating four allosteric sites--the implication is that the subunits of a given isozyme of pyruvate kinase are functionally identical, each having a binding site for all molecules that are bound to that isozyme of pyruvate kinase. In other words,

there are probably four binding sites per tetramer of pyruvate kinase, no matter which ligand is considered--substrates (ADP, ATP, P-enol-pyruvate, pyruvate), cofactors (monovalent and divalent cations), and effectors (e. g., fructose 1, 6-diphosphate, various amino acids).

### Bovine Pyruvate Kinase Isozymes

The isozyme distribution in bovine tissues is superficially the same as that reported earlier for rat tissues: type M subunits are present in large amounts in skeletal muscle, heart, and brain; type L is found in liver, kidney, and erythrocytes; and type K is synthesized in appreciable quantities in all adult tissues except striated muscle. However, additional, intermediate bands are seen.

The identification of these intermediate bands as hybrids is confirmed by isolation, dissociation, and reassociation of  $K_2L_2$  and  $K_3M$  from bovine kidney. Such treatment of separated hybrids resulted in regeneration of new hybrid sets. Under normal, nondenaturing conditions, the isolated hybrids showed no tendency to interconvert or to give rise to additional electrophoretic forms, thereby virtually precluding the possibility that the intermediate bands are interconvertible species. All the bands seen in bovine tissues are accounted for in terms of three parental isozymes and their hybrid tetramers, making it unnecessary to associate any of the intermediate bands with additional isozymes or non-tetrameric forms of the three major



isozymes.

The tissue distribution of pyruvate kinase isozymes is therefore more complex than previously reported, due to the widespread existence of the hybrids. In bovine kidney, for example, there are three K-L hybrids ( $K_3L$ ,  $K_2L_2$ , and  $KL_3$ ) in amounts that suggest assembly from a pool of roughly equal quantities of K and L subunits. In addition, however, there are also substantial quantities of K-M hybrids, the quantity of which indicates that cells making K-M hybrids are either more numerous than those making K-L hybrids or that their pyruvate kinase activity is greater. Note, by way of comparison, that K-L hybrids were not detected in liver, consistent with the result of cell isolation studies with rodents showing that the type L isozyme is found in liver parenchymal cells and the type K isozyme in non-parenchymal cells (Van Berkel et al., 1972; Crisp and Pogson, 1972).

Electrophoretic patterns that appear to be K-L hybrid sets have been reported in small intestine and kidney (Imamura and Tanaka, 1972; Whittell et al., 1973; Osterman and Fritz, 1974), but aside from fetal and newborn mammals, K-M sets have been reported only in spleen and testis (Whittell et al., 1973). In our studies,  $K_3M$  was found in all tissues examined that also had  $K_4$ .  $K_2M_2$  and  $KM_3$  were detectable in several of these tissues and were presumably present at undetectable levels in the others.  $M_4$  was not seen on the zymograms in any of the tissues that contain predominantly type K subunits, but

since M subunits must be synthesized in order to produce K-M hybrids, one must conclude that small amounts of  $M_4$  are present at levels too low to detect. Skeletal and cardiac muscles appear to contain a small amount of  $KM_3$ , making it probable that type K subunits are produced even in striated muscle, which had previously been thought to contain only the  $M_4$  isozyme. In general, K-M hybrids are apparently more pronounced in bovine tissues than in rat tissues. The adult mouse liver used in studying the electrophoretic mobilities of the mouse pyruvate kinase isozymes had a strong band corresponding to  $K_3M$ , and the presence of K-M hybrids to an extent comparable to the bovine tissues has recently been shown in mouse tissues (Tolle *et al.*, 1976).

Thus, the pattern that emerges from this study is that some type M subunits are produced in tissues that were thought to make only  $K_4$ , and some type K subunits are produced in tissues that were presumed to make only type M subunits, resulting in a complex, tissue-specific pattern of hybrid isozymes. Such hybrids are presumably a result of association of two subunit types synthesized within a single cell.

The functional significance of the hybrid isozymes is not really clear. However, it was shown that in the case of the L-M hybrids (formed *in vitro*), the  $L_3M$  tetramer has kinetics virtually indistinguishable from  $L_4$ , while the kinetics of  $LM_3$  closely resemble those

of  $M_4$  (Dyson and Cardenas, 1973). If this observation can be generalized to the K-L and K-M hybrids, then there would appear to be little or no advantage in having, for example, pure  $K_4$  in a cell as opposed to having  $K_4$  and  $K_3M$ . Hence, a mechanism for absolute repression of the unwanted isozymes may not really be necessary.

### Chicken Pyruvate Kinase Isozymes

Only two different bands of pyruvate kinase activity are detectable after electrophoresis of extracts of a variety of adult and fetal chicken tissues. These bands are reproducibly distinguishable from each other, and there is no evidence that they are interconvertible. These two chicken isozymes have been given the same designations as have been used in describing two of the mammalian isozymes: type M for the pyruvate kinase found in skeletal muscle and type K for the form in kidney. Chicken type M pyruvate kinase corresponds in its overall characteristics to mammalian type M and chicken type K corresponds to mammalian type K. The results indicate that an isozyme corresponding to the major mammalian liver isozyme (type L) does not occur in chickens.

The first point of correspondence between chicken and mammalian isozymes lies in electrophoretic mobility. Type K is the most cathodic isozyme in each case. Type M migrates to a position not greatly removed from type K.

Chicken type K pyruvate kinase has a pattern of tissue distribution that is similar in many respects to that of mammalian type K. Chicken type K appears in all extracts of adult tissues except skeletal muscle and heart and is the only band seen in the whole 7-day embryo. It is also a minor constituent of the brain.

The tissue distribution of chicken type M and mammalian type M are also similar, both being the sole isozyme found in adult muscle and the major isozyme in brain. The transition from K to M in fetal chicken skeletal muscle parallels equivalent isozyme shifts in the fetal rat (Osterman et al., 1973).

There is no evidence in the zymograms for a chicken pyruvate kinase corresponding to the mammalian type L isozyme. Chicken liver, kidney, and jejunum appear to have only the type K isozyme. Similarly, whereas mammalian erythrocytes probably have the type L isozyme (Bigley et al., 1968; Nakashima et al., 1974; Cardenas et al., 1975a), chicken erythrocytes have type K. Thus it seems that where type L is found in mammals, type K alone is instead found in the corresponding chicken tissues.

While it is true that the type L isozyme seems to be the most unstable of the mammalian isozymes, if an isozyme corresponding to the mammalian type L exists in chicken tissues, it should have been seen using the conditions of extraction and electrophoresis employed, since we are able in this laboratory to detect type L pyruvate kinase

in bovine, rat, mouse, and human tissues utilizing the same procedures.

In addition to similarities in tissue distribution, chicken type M and type K have kinetic properties corresponding to those of mammalian types M and K respectively. Type M from adult chicken breast muscle, like mammalian M, has hyperbolic kinetics with respect to P-enolpyruvate concentration and is not activated by fructose 1,6-diphosphate (Cardenas et al., 1975b). Chicken type K, on the other hand, has sigmoidal kinetics and is activated by fructose 1,6-diphosphate to give a hyperbolic curve, resembling the mammalian type K pyruvate kinase purified by Imamura et al. (1972).

Additional evidence for the absence of a chicken type L isozyme comes from the lack of substrate inhibition by ADP of pyruvate kinase activity in liver. Since mammalian type L pyruvate kinase is inhibited by high concentrations of ADP (Tanaka et al., 1967; Kutzbach et al., 1973; Cardenas et al., 1975a), the pyruvate kinase isozyme content of avian liver must differ from that of mammalian liver.

The results of the immunological studies also indicate the absence in chicken liver of an isozyme corresponding to the mammalian type L isozyme. In the rat, type K and type M are very close immunologically, but type L does not cross-react with either (Imamura et al., 1972). However, antiserum prepared against bovine or chicken type M pyruvate kinase completely inactivates the

pyruvate kinase activity in adult chicken liver, as well as the activity of the isozyme in extracts of fetal thigh muscle (type K). Assuming there are no complicating factors affecting this analysis, chicken liver must therefore contain all of its pyruvate kinase in the form of type K and none in a form immunologically comparable to the type L isozyme.

The presence in chicken liver of only one pyruvate kinase isozyme, which seems to correspond to the mammalian type K, is interesting in light of observations that the level of hepatic pyruvate kinase activity does not change under different dietary conditions in the pigeon (Gevers, 1967) or the chicken (Pearce, 1971) whereas the level of rat type L pyruvate kinase, but not type K, is subject to dietary control in liver and kidney (Tanaka *et al.*, 1967; Sandoval and Carbonell, 1973). Thus, in this respect also chicken type K is similar to mammalian type K, which is not subject to dietary control.

The kinetics observed for pyruvate kinase in extracts of adult chicken liver and of fetal thigh muscle are quite similar to each other. Extracts from adult chicken liver and from fetal thigh both had sigmoidal kinetics with P-enolpyruvate, but measurements of the Hill coefficients gave a value of 2.1 for pyruvate kinase from chicken liver, which is within experimental error of the value (1.9) obtained for the (type K) pyruvate kinase from chicken fetal thigh muscle.

There are, however, differences in the  $S_{0.5}$  for P-enolpyruvate when

the pyruvate kinases in crude extracts from fetal thigh and adult chicken liver are compared. The values were 0.33 mM for adult liver and 0.20 mM for fetal thigh muscle (see Figs. 15 and 16). This difference was reproduced when the extracts were prepared by any of the procedures described in the section on Materials and Methods, including ammonium sulfate precipitation. Nor did incubation of the fetal thigh muscle extract with EDTA shift the kinetic curve toward that of adult liver. Such a shift would be expected if there were interconvertible forms of the kind described for rat liver cells in culture (Walker and Potter, 1973) and for rat adipose tissue (Pogson, 1968a,b).

However, the kinetic difference between the pyruvate kinase in fetal thigh muscle and adult liver must be interpreted with caution because of the experimental difficulties involved in performing kinetic studies on crude extracts. The differences could conceivably be due to the occurrence of large background reactions from contaminating enzymes in liver or to effectors left bound to the enzymes despite dialysis, gel filtration, or precipitation of the proteins with ammonium sulfate. In addition, the possibility of an endogenous, non-dialyzable inhibitor in adult liver, such as that suggested for rabbit skeletal muscle pyruvate kinase by Bondar and Pon (1969), cannot be ruled out.

In distinguishing among unpurified isozymes, greater confidence can probably be placed on the electrophoretic and immunological

results than on the kinetic studies. Therefore, it appears that the pyruvate kinase isozymes in fetal thigh muscle of chicken and in adult chicken liver are probably identical, and that chicken liver contains only one isozyme.

The conclusion that only two isozymes of pyruvate kinase, corresponding to the mammalian types K and M, exist in chicken tissues has recently been corroborated by the isoelectric focusing and kinetic studies of pyruvate kinase in chicken tissues done by Ibsen et al. (1976). The same laboratory has also investigated the inhibition of the chicken isozymes by amino acids (Ibsen and Marles, 1976).

With the possible exception of erythrocytes, the isozyme content of which remains controversial, mammalian type L pyruvate kinase is found only in those tissues where gluconeogenesis is thought to be most important. The implication is that the properties of this enzyme are especially adapted to support gluconeogenesis. Since chicken type K has a higher Hill coefficient than mammalian type K (1.9 vs. 1.5), it already has many of the advantages of the mammalian type L pyruvate kinase. One can speculate, therefore, that gluconeogenesis in chicken tissues may be adequately supported by type K without the need for a specialized isozyme, and that the properties of chicken type K represent a compromise between the characteristics needed for gluconeogenic and non-gluconeogenic tissues.



### Bovine Type $K_4$ Pyruvate Kinase

The principal difficulty in isolating type  $K_4$  pyruvate kinase from bovine tissues is the presence of substantial quantities of the  $K_3M$  hybrid in all adult tissues examined which contain  $K_4$ . The separation of  $K_3M$  from  $K_4$  on CM-Sephadex columns is only slight, resulting in poor yields of  $K_4$  free of contaminating  $K_3M$ . This small separation of  $K_3M$  from  $K_4$  is hardly surprising in light of the apparently small difference in isoelectric pH--8.9 for  $M_4$  and 9.0 for  $K_4$ . This small difference in charge between the M and K subunits is reflected in the long electrophoresis times required to separate  $M_4$  and  $K_4$  adequately in order to see the K-M hybrids. The poor yield of  $K_4$  free of  $K_3M$  has made it difficult to effect further purification of the  $K_4$  preparations, and without further purification physical characterization of the  $K_4$  pyruvate kinase is not possible.

Several types of experiments have been performed on the partially purified preparations of type  $K_4$  pyruvate kinase from bovine kidney. The velocity of the enzyme as a function of P-enolpyruvate concentration is slightly sigmoidal, resembling a number of the kinetic profiles reported for type  $K_4$  from other mammalian sources. Compared to the kinetic profile reported for the only completely purified  $K_4$  pyruvate kinase (isolated from Yoshida ascites rat hepatoma by Imamura et al., 1972), the bovine  $K_4$  has approximately the same

Hill coefficient (around 1.5 in each case) but a higher  $S_{0.5}$  for P-enolpyruvate (1.1 vs. 0.4 mM). In addition, fructose 1,6-diphosphate decreases the  $S_{0.5}$  of the bovine  $K_4$  only slightly, while it decreases that of the purified rat hepatoma  $K_4$  substantially. Comparing the bovine  $K_4$  kinetic profile to that of partially purified rat kidney cortex  $K_4$  pyruvate kinase, one finds that the bovine results fall within the range of the unpublished rat results. The rat  $K_4$  prepared by isoelectric focusing by Ibsen and Trippet (1973) has kinetic parameters similar to the rat ascites hepatoma  $K_4$  of Imamura et al. (1972). In contrast, the rat  $K_4$  prepared by ion-exchange chromatography by Jiménez de Asúa et al. (1971b) and by Costa et al. (1972) has a higher  $S_{0.5}$  for P-enolpyruvate (2.1-2.2 mM) than the bovine  $K_4$  and no change at all in the velocity profile in the presence of fructose 1,6-diphosphate. The velocity profile of the bovine  $K_4$  is not actually identical to any of the profiles for  $K_4$  from mammalian sources listed in Table II.

The  $S_{0.5}$  for P-enolpyruvate of the bovine embryo extract (0.66 mM) is close to the  $S_{0.5}$  values of rat  $K_4$  reported by Imamura et al. (1972) and by Ibsen and Trippet (1973), but is probably significantly different from the  $S_{0.5}$  for the partially purified bovine  $K_4$ . This difference may reflect a change in the kidney  $K_4$  during the isolation, resulting in a raising of the  $S_{0.5}$ .

The strong inhibition of the bovine type  $K_4$  pyruvate kinase by

low levels of L-phenylalanine and L-alanine is consistent with the results for most type  $K_4$  preparations from other mammals (Table III). The inhibition is reversed, at least at the lower amino acid concentrations, by fructose 1,6-diphosphate, which is consistent with the results for many, but not all, the  $K_4$  studies listed in Table III.

Cardenas et al. (1975c) have reported the effects of L-phenylalanine and L-alanine on bovine type L and type M pyruvate kinases as well. The inhibition of type L by L-phenylalanine is comparable to that of type K but not quite as strong; the  $K_i$  for type L at 0.5 mM P-enolpyruvate is 0.49 mM compared to the  $K_i$  for type K of 0.11 mM. Type M is inhibited by higher concentrations of L-phenylalanine; the  $K_i$  is 22.0 mM at 0.5 mM P-enolpyruvate. The addition of 5 mM L-alanine reversed the L-phenylalanine inhibition of type M but had no effect on the L-phenylalanine inhibition of type L.

Bovine type L pyruvate kinase differs from the corresponding rat isozyme with respect to inhibition by L-alanine (Cardenas et al., 1975c). While rat type L is strongly inhibited by this amino acid, the bovine type L is scarcely affected. In this respect bovine type L is more similar to guinea pig type L, which is only moderately inhibited by L-alanine (Faulkner and Jones, 1975a).

Thus, the three bovine isozymes show distinctive differences from one another in their kinetic properties with L-alanine. Type M is not inhibited by L-alanine, but L-alanine reverses the inhibition

caused by L-phenylalanine. Type L is not inhibited by L-alanine either, but L-alanine does not reverse its inhibition by L-phenylalanine. Type K, on the other hand, is strongly inhibited by L-alanine as well as L-phenylalanine.

The inhibition of pyruvate kinase isozymes by L-phenylalanine and L-alanine suggests that these effects may play a role in metabolic regulation of the activity of pyruvate kinase, particularly of type  $K_4$ . Since L-alanine is the transamination product of pyruvate, it could exert a feedback control. Van Berkel (1974) has concluded that at physiological concentrations of L-alanine,  $Mg^{2+}$ , ADP, and P-enolpyruvate, the type K pyruvate kinase activity of rat liver is mainly dependent upon the fructose 1, 6-diphosphate concentration.

The bovine  $K_4$  preparation has also been used to confirm the immunological cross-reactivity of type K and type M pyruvate kinase originally reported by Imamura et al. (1972). Antiserum prepared by inoculation of chickens with bovine  $M_4$  pyruvate kinase neutralized  $K_4$  almost as well as  $M_4$  itself.

The properties of the partially purified bovine kidney  $K_4$  pyruvate kinase are thus similar or identical to the properties of other mammalian  $K_4$  pyruvate kinases. The parameters of the kinetic profile with respect to P-enolpyruvate are not identical to any of those reported in the literature, but due to the great variability in published values, lack of exact agreement is not surprising.

### Conclusion

The view of pyruvate kinase that emerges from the literature and from the findings of this thesis is that of a tetrameric enzyme, existing in mammals as at least three distinct isozymes. The subunits of a given isozyme are probably functionally identical, having a binding site for all molecules that are bound to that isozyme of pyruvate kinase. One isozyme (type K) seems likely to be the sole isozyme in the early fetus and continues to be synthesized in some degree in most or all adult bovine tissues. Two differentiated types of pyruvate kinase--type M and type L--are adapted for specialized functions in highly glycolytic and in gluconeogenic tissues, respectively. The kinetic properties of type K seem to be intermediate between those of the differentiated types. Since type K subunits seem to be produced in cells containing mainly type M, and type M subunits are present in many tissues other than striated muscle and brain, where they are most abundant, it seems that absolute repression of unselected K or M isozymes in K- or M-containing bovine tissues does not occur. This pattern may be true to varying extents for most mammals.

In chickens, the overall pattern of tissue distribution and kinetic properties is the same as in mammals, with the exception that only two isozymes exist. Chickens contain type K and type M pyruvate kinases but do not have an isozyme analogous to the mammalian type L.

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