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Effects of Phenylalanine and Alanine on the Kinetics of Bovine Pyruvate Kinase Isozymes†

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ABSTRACT: The effects of L-phenylalanine and of L-alanine on the kinetics of the three bovine pyruvate kinase isozymes have been determined. All three isozymes are inhibited by L-phenylalanine, with K_i's at 0.5 m*M* phosphoenolpyruvate of 0.11 m*M* for type K, 0.49 m*M* for type L, and 22.0 m*M* for type M. Only type K was inhibited by L-alanine, and its K_i was 0.042 m*M*. The addition of 5 m*M* L-alanine to the assay mixture reversed L-phenylalanine inhibition of type M but had no effect on the L-phenylalanine inhibition of type L. Using partially purified rat enzymes, we were able to confirm that both rat types K and L pyruvate kinases are inhibited by L-alanine, in contrast to bovine type L, which is not significantly inhibited by this amino acid. Furthermore, the bovine isozymes can be easily distinguished from one another by their kinetic properties in the presence of L-alanine and/or L-phenylalanine; the susceptibility of the isozymes, especially types L and K, to inhibition by amino acids provides a possible mechanism for regulating pyruvate kinase activity.

Three distinct isozymes of pyruvate kinase are known to exist in mammalian tissues (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Whittell et al., 1973; Ibsen and Trippet, 1973; Carbonell et al., 1973; Farina et al., 1974; Cardenas et al., 1975). Type K is found in all early fetal tissues and in most adult tissues. Type M is found mainly in brain and muscle, while type L occurs mainly in liver, kidney, and intestinal mucosa.[1] Erythrocytes have

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[1] Under the IUB system of nomenclature, types L, M, and K would be called I, II, and III, respectively. Other systems of nomenclature have referred to type L as PyK B; type M as M_1 or PyK A; and type K as M_2 or PyK C. Since hybridization of three subunit types could produce a total of 15 electrophoretic forms, use of the IUB system of nomenclature would be rather complicated. Thus, we have chosen to use the K-L-M mnemonic system described here.

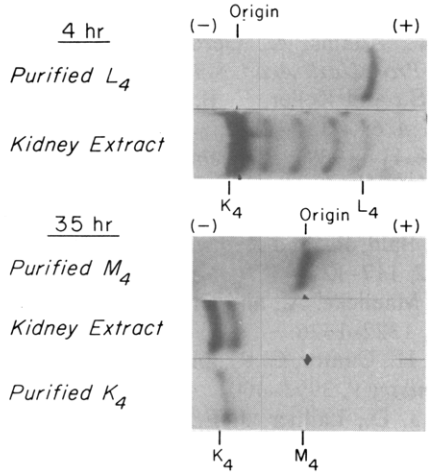

FIGURE 1: Electrophoresis on cellulose acetate of bovine kidney extracts and of purified bovine pyruvate kinase isozymes. Electrophoresis of kidney extracts was continued for 4 and for 35 hr in order to visualize both the K–L and K–M hybrid sets present in this tissue. Experimental procedures are described in the text.

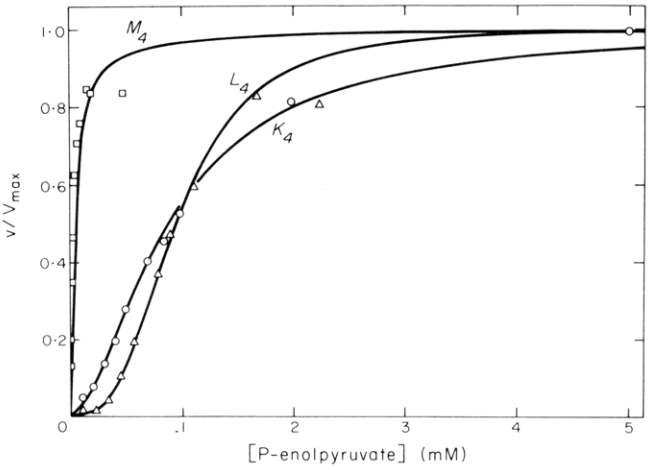

FIGURE 2: Activity of bovine pyruvate kinase isozymes as a function of P-enolpyruvate. Assay conditions are described in the Experimental Procedures.

also been reported to contain type L (Bigley et al., 1968) although more recent work suggests that erythrocyte pyruvate kinase may represent either a fourth isozyme type or a hybrid (Imamura and Tanaka, 1972; Whittell et al., 1973; Nakashima et al., 1974). Several tissues appear to contain natural hybrids of two or more of the isozyme types described above. This apparently occurs when more than one isozyme is synthesized simultaneously in the same cell. Under standard assay conditions, type M pyruvate kinase has hyperbolic kinetics with P-enolpyruvate,[2] while both types K and L have sigmoidal kinetics with this substrate.

Carminatti et al. (1971) and Kayne and Price (1973) showed that rabbit type M pyruvate kinase is allosterically inhibited by Phe. The velocity plot is sigmoidal with respect to Phe concentration, but plots of velocity with respect to P-enolpyruvate retain their hyperbolic profile in the presence of Phe. The inhibition was reversed by Cys, Ala, and Ser. Cardenas et al. (1975) recently showed bovine type M pyruvate kinase to have a sigmoidal velocity profile when P-enolpyruvate is varied at constant Phe concentrations.

Others have studied the effects of Ala and of Phe mainly on partially purified types K and L pyruvate kinases or on tissue extracts containing these isozymes. Llorente et al. (1970) reported that Ala and ATP inhibit pyruvate kinase in extracts from rat liver or kidney, and Stifel and Herman (1971) reported inhibition of rat jejunal pyruvate kinase by both Ala and Phe, with K_i's of 0.75 mM and 0.4 mM, respectively. Carbonell et al. (1973) observed Ala, Cys, and Phe inhibition of pyruvate kinase in rat liver extracts, in agreement with Jiménez de Asúa et al. (1971), who found that Phe and Ala inhibited both pyruvate kinase isozymes of rat liver. Black and Henderson (1972) observed inhibition by Ala and Phe on human erythrocyte pyruvate kinase, while Balinsky et al. (1973) found that Ala inhibits the isozymes found in human liver samples, with K_i's at 0.5 mM P-enolpyruvate of 0.63 mM for normal liver pyruvate kinase, 0.52 mM for the enzyme from hepatoma, and 0.40 mM for the enzyme in fetal liver. Ibsen and Trippet (1974) measured the effects of these amino acids on rat pyruvate kinase isozymes separated by isoelectric focusing. They found considerable inhibition of type K by either Ala or Phe and strong inhibition of type L by Ala but less inhibition by Phe. The only comprehensive study performed to date with purified isozymes was that of Imamura et al. (1972), who studied the three isozymes from the rat. At unstated P-enolpyruvate concentrations, they found K_i's for Ala of 0.6 mM for type K and 1.0 mM for type L. For Phe, the K_i's were 0.5 mM for type K and 5 mM for type L. They found no significant inhibition of type M by either amino acid.

While Phe appears to inhibit all three mammalian pyruvate kinases, Ala seems to inhibit human and rat types K and L but reverses Phe inhibition of rabbit type M. However, considerable variation in the reported extent of inhibition by these two amino acids is evident among literature values. Such variation is not surprising for two reasons. Firstly, with the experimental protocols used, apparent K_i's are profoundly affected by P-enolpyruvate concentrations and by the absence or presence of Fru-1,6-P_2. Secondly, most of the samples used for kinetic analyses were not analyzed in order to establish their pyruvate kinase isozyme content. Such analysis is very important since multiple pyruvate kinase isozymes and isozyme hybrids occur in many tissues (Susor, 1970; Susor and Rutter, 1971; Whittell et al. 1973; Strandholm et al., 1975).

In the present work we have used bovine pyruvate kinase isozymes which have been carefully separated from other pyruvate kinase isozymes and hybrids in order to study the relative effects of Ala and/or Phe on the three pyruvate kinase isozymes from bovine tissues, and we compare the results with those obtained for rat types K and L under identical conditions. While all three bovine isozymes are inhibited by Phe, only type K is inhibited to a significant extent by Ala.

Experimental Procedures

Materials. Distilled, deionized water was used for making all solutions. KCl and $MgCl_2$ were standard reagent grade. Substrates, lactate dehydrogenase, Phe, and Ala were obtained from Sigma Chemical Co. Bovine skeletal muscle and liver pyruvate kinases were prepared according to procedures described in Cardenas et al. (1973) and Cardenas and Dyson (1973). Types L and K pyruvate kinases were prepared from rat liver through the DEAE chroma-

[2] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; Fru-1,6-P_2, fructose 1,6-bisphosphate; amino acid abbreviations are consistent with those recommended by the International Union of Biochemistry, e.g., Ala for L-alanine and Phe for L-phenylalanine.

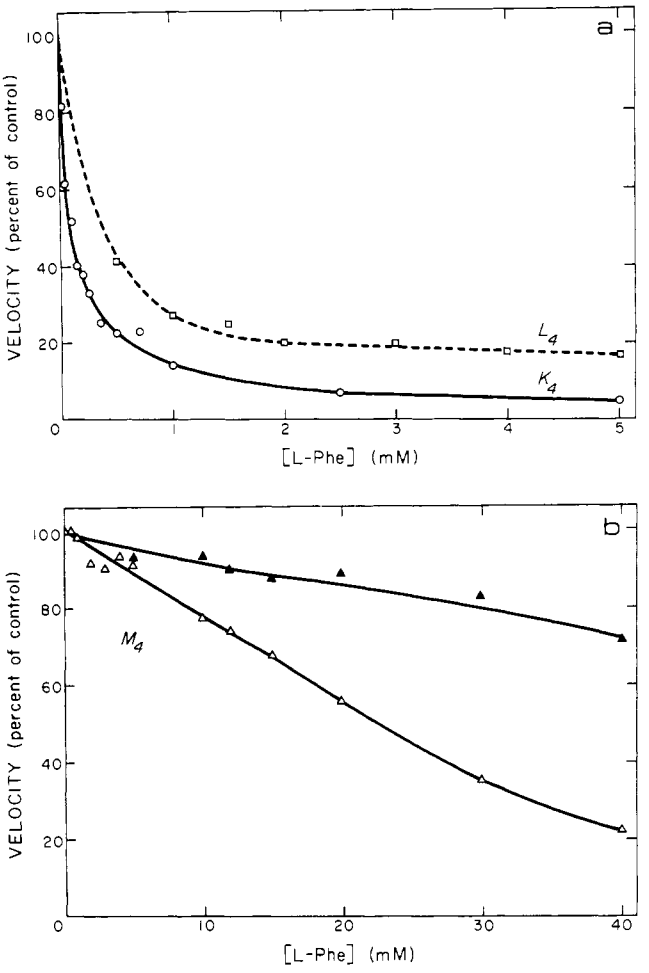

FIGURE 3: Activity of bovine pyruvate kinase isozymes as influenced by varying concentrations of Phe at 0.5 m*M* P-enolpyruvate. (○) K_4, (□) L_4; (△) M_4 in the absence of Ala; (▲) M_4 in the presence of 5 m*M* Ala. Note the different scales for Phe concentrations in a and b.

tography step with the same procedure as used for the bovine type L isozyme (Cardenas and Dyson, 1973). The rat type K isozyme was eluted in the void volume of the DEAE column, while the type L isozyme was eluted with a 0–0.3 *M* KCl gradient.

Bovine type K (K_4) pyruvate kinase was prepared from kidney by a procedure similar to that used for type M from skeletal muscle (Cardenas et al., 1973) but with the following modifications. The ground kidney was homogenized with 0.05 *M* Tris-HCl (pH 7.5), 0.15 *M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, and 10 m*M* β-mercaptoethanol. After centrifugation, the extract was filtered through several layers of cheesecloth. During the heat step, the extract was held at 60° for only 4 min. Ammonium sulfate fractionation was 46–60% (270 g/l. followed by 90 g/l.) at 0°. The CM-Sephadex C-50 column was equilibrated and run in 0.04 *M* imidazole-acetic acid (pH 6.5 at 4°), 0.5 *M* sucrose, 1 m*M* EDTA, and 10 m*M* β-mercaptoethanol, and at a flow rate of 30 ml/hr. The identity and homogeneity of all pyruvate kinase isozymes was checked by electrophoresis on cellulose acetate using the activity detection procedure of Susor and Rutter (1971) as modified by Cardenas and Dyson (1973). Bovine liver extracts were found to contain, in addition to K_4 and L_4, small quantities of K–M hybrids. Bovine kidney contains mainly K_4 but also has substantial amounts of both K–M and K–L hybrids (see Figure 1). Using the CM-Sephadex column described above, type K_4 was separated from the other species of pyruvate kinase by its later elution from this column. The specific activities of bovine K_4 preparations were usually around 20 units (micromoles per minute) of pyruvate kinase per mg of protein. Although the rat type L preparation and the bovine and rat type K preparations still contained other proteins, the samples were shown by electrophoresis to be homogeneous with respect to pyruvate kinase activity, with no appreciable contamination by hybrids or by other pyruvate kinase isozymes (see Figure 1).

Table 1: Inhibition Constants (K_i) for Pyruvate Kinase Isozymes Determined at 0.5 m*M* P-enolpyruvate.

Pyruvate Kinase Isozyme	K_i (m*M*)	
	Phe	Ala
Bovine K_4	0.11	0.042
Bovine L_4	0.49	*a*
Bovine M_4	17.0	*a*
Rat K_4	0.5[b]	0.2
Rat L_4	5[b]	0.1

[a] No inhibition was observed. [b] From Imamura et al. (1972).

Enzyme assays were performed using the coupled assay procedure of Bücher and Pfleiderer (1955) on a Beckman Acta III spectrophotometer equipped with an automatic sample changer. Assay mixtures consisted of 1 ml of 0.05 *M* imidazole-HCl buffer (pH 7.5) with 0.1 *M* KCl, 10 m*M* $MgCl_2$, 2 m*M* ADP, 0.5 m*M* P-enolpyruvate unless otherwise indicated, 0.16 m*M* NADH, and approximately 20 units (micromoles per minute) of lactate dehydrogenase. The temperature was regulated at 25° P-enolpyruvate concentrations of stock solutions were determined enzymatically by adding pyruvate kinase to a cuvet containing a limiting amount of P-enolpyruvate and all the other components necessary for the reaction, then determining the total change in absorbance at 340 nm.

Due to the allosteric nature of most of the kinetics in this study, values of K_i reported here are the concentrations of inhibitor required to produce 50% inhibition at 0.5 m*M* P-enolpyruvate.

Results

The velocity profiles of bovine types K, L, and M pyruvate kinase as a function of P-enolpyruvate concentration are shown in Figure 2. The effects of various levels of Phe on the kinetics of bovine isozymes at 0.5 m*M* P-enolpyruvate are shown in Figure 3 and the calculated values of K_i are listed in Table I. Phe was found to inhibit all three isozymes, causing the greatest extent of inhibition in type K and the least extent in type M.

Addition of 5 m*M* Phe to the assay medium changed the velocity curve of type M pyruvate kinase with respect to P-enolpyruvate from hyperbolic to sigmoidal (see Figure 4a) with a concomitant increase in the Hill coefficient from 1.0 to 1.9. The results obtained for the effect of Phe on bovine type M pyruvate kinase are consistent with studies previously reported by Carminatti et al. (1971) and by Kayne and Price (1973) for the rabbit skeletal muscle enzyme and are in agreement with their hypothesis that Phe inhibits type M pyruvate kinase by binding at an allosteric site.

Figure 4b shows the kinetics of bovine type L in the absence and presence of 5 m*M* Phe. This isozyme normally has sigmoidal kinetics with P-enolpyruvate. Phe shifts the sigmoidal curve toward higher P-enolpyruvate concentra-

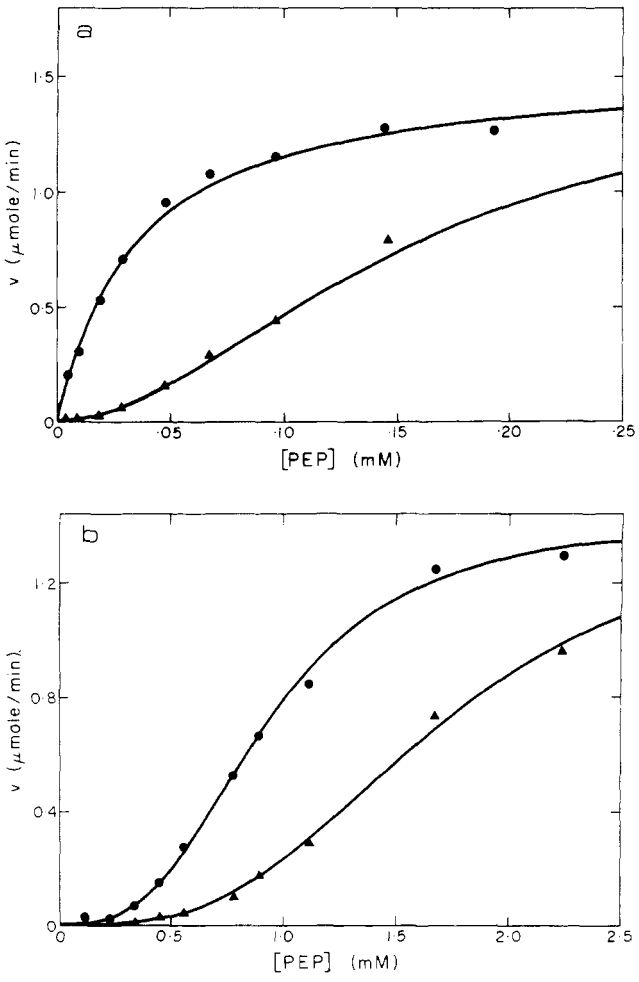

FIGURE 4: The effect of Phe on the kinetics of bovine types M (a) and L (b) pyruvate kinases as a function of P-enolpyruvate concentration. (●) Velocities in the absence of Phe; (▲) velocities in the presence of 5 m*M* Phe.

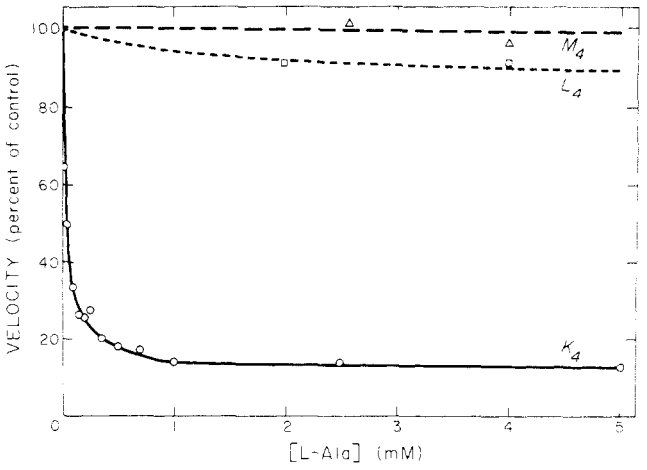

FIGURE 5: The effect of Ala on bovine pyruvate kinase isozymes at 0.5 m*M* P-enolpyruvate.

tions, with the $S_{1/2}$ for P-enolpyruvate increased from 0.9 m*M* in the absence of Phe to 1.7 m*M* in the presence of 5 m*M* Phe. However, the Hill coefficient increases only slightly, with 3.0 and 3.1 being the respective values obtained in the absence and presence of Phe. With either type M or type L pyruvate kinase, the inhibiting effects of Phe can be largely reversed by the addition of Fru-1,6-P_2, the allosteric activator, to the assay medium, and the velocity profiles become hyperbolic.

Shown in Figure 5 are the effects of various concentrations of Ala on the three isozymes at 0.5 m*M* P-enolpyruvate. Under these conditions, Ala has little or no effect on bovine type L or type M pyruvate kinase but strongly inhibits type K, with a K_i of 0.042 m*M*. Addition of 1 m*M* Fru-1,6-P_2 reverses the Ala inhibition of the type K enzyme. When bovine liver extracts are tested for sensitivity to inhibition by Ala, only a small amount of inhibition was observed which was proportional to the amcunt of type K in the liver extracts. Thus, purification of bovine type L pyruvate kinase does not appear to change its susceptibility to inhibition by Ala.

We also measured the effect of Ala on partially purified types K and L pyruvate kinases from rats in order to determine whether the apparent lack of inhibition of bovine type L by Ala might be due to different experimental procedures from those of others. In qualitative agreement with previous reports, we found Ala to be a strong inhibitor of rat types K and L pyruvate kinases, with K_i values of 0.2 and 0.1 m*M*, respectively. Thus, rat and bovine type L pyruvate kinases appear to differ in their kinetic response to Ala, since the former is inhibited to a considerable degree while the latter is essentially unaffected by Ala at any of the many P-enolpyruvate concentrations used in this study.

The presence of 5 m*M* Ala reverses Phe-induced inhibition of type M pyruvate kinase (Figure 3b) but has little effect on the kinetics of type L. Thus, the three bovine isozymes differ considerably from one another in their kinetics with substrates in the presence or absence of Phe and/or Ala: in the absence of free amino acids, type M has hyperbolic kinetics with P-enolpyruvate, while type K and L both have sigmoidal kinetics with this substrate. Ala itself inhibits its only type K pyruvate kinase, but it reverses Phe induced inhibition only with type M.

Discussion

All three bovine pyruvate kinase isozymes are inhibited by Phe but their sensitivities to this amino acid vary considerably. Type K is by far the most sensitive, with a K_i of 0.11 m*M* at 0.5 m*M* P-enolpyruvate. Type L is inhibited somewhat less strongly, with a K_i of about 0.5 m*M*. Type M pyruvate kinase is also inhibited but to a much lesser extent, since its K_i for Phe is 22 m*M* at 0.5 m*M* P-enolpyruvate. The apparent K_i for Phe is very profoundly affected by the P-enolpyruvate concentration. For instance, the K_i of M_4 for Phe is 22 m*M* at 0.5 m*M* P-enolpyruvate, 12 m*M* at 0.3 m*M* P-enolpyruvate, but only 3.3 m*M* at 0.1 m*M* P-enolpyruvate. This trend is in qualitative agreement with the results of Carminatti et al. (1971) for rabbit skeletal muscle pyruvate kinase.

The three bovine pyruvate kinase isozymes exhibit striking differences from one another in their kinetic behavior with Ala. Type K is strongly inhibited by Ala and has a K_i of 0.042 m*M* at 0.5 m*M* P-enolpyruvate. Type L is only slightly inhibited by Ala, but its inhibition by Phe is not reversed by Ala. Type M is not inhibited by Ala, but 5 m*M* Ala reverses the inhibition caused by Phe.

Bovine and rat pyruvate kinase isozymes appear to be similar in many respects, but the two type L isozymes show very different kinetic responses toward Ala. While the rat type L enzyme is strongly inhibited by this amino acid, the equivalent enzyme from bovine tissues is essentially unaf-

fected. The reason for this difference between the two mammalian type L isozymes is unknown.

The striking effects of Phe and Ala on bovine pyruvate kinase isozymes suggest a possible role for these amino acids in regulating catalytic activity, especially in the case of type K, which has K_i's for Ala and Phe well below the concentrations normally found in mammalian tissues. For instance, Ala concentrations in liver are reported to fall in the range of 1–4 m*M* (Burns et al., 1970), while those of Phe are found to be 0.07–0.15 m*M* (Kirsten et al., 1961). Conceivably, Ala could be exerting control as a feedback inhibitor of type K or L pyruvate kinase, since most of the Ala synthesized in a cell is produced by transamination of pyruvate. In the absence of significant levels of Fru-1,6-P_2, high levels of either Phe or Ala might cause considerable inhibition of pyruvate kinase and hence of glycolysis, particularly in tissues containing a large proportion of type K or type L pyruvate kinase. In a recent paper, Van Berkel (1974) reported that at physiological levels of Ala, Mg^{2+}, ADP, and P-enolpyruvate, type K pyruvate kinase activity is dependent on the Fru-1,6-P_2 concentration.

Genetic deficiencies resulting in primary elevation of blood Ala concentrations have not been observed. However, histidinemia, citrullinemia, and lactic acidosis are associated with significant elevations of Ala, although these are probably secondary effects. Weber (1969a,b) proposed that the brain damage occurring in phenylketonuria might result from the inhibitory effects of high Phe levels on brain pyruvate kinase. If such a model is true, high levels of Phe would be especially damaging in tissues with a high proportion of type K pyruvate kinase, a situation which probably occurs in young, developing animals. Information obtained with rat and bovine pyruvate kinases suggest the importance of determining isozyme patterns at various developmental stages, particularly of nerve and brain tissue, and of performing extensive studies of the effects of amino acids on human pyruvate kinases.

Acknowledgments

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