Pyruvate Kinase Isozymes in Adult and Fetal Tissues of Chicken†

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ABSTRACT: Tissues of fetal and adult chickens were examined for pyruvate kinase activity. Two electrophoretically distinguishable and noninterconvertible isozymes were found. One of these, designated as type K (for kidney), is the sole pyruvate kinase in the early fetus and is found in appreciable quantities in all adult tissues except striated muscle. The second isozyme, type M, appears shortly before hatching in striated muscle and brain. These two isozymes correspond in their developmental pattern, tissue distribution, electrophoretic, immunological, and kinetic properties to similarly designated mammalian pyruvate kinases. However, no kinetic, immunological, or electrophoretic evidence could be found for a chicken isozyme corresponding to the mammalian type L pyruvate kinase. As the latter isozyme seems to be limited in its distribution mostly to highly differentiated gluconeogenic tissues (notably liver, kidney, and small intestine), our results support the proposition that the mammalian type L pyruvate kinase is a specialized isozyme that is present in mammals but not in birds.

Three electrophoretically distinct and noninterconvertible isozymes of pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40) have been identified 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., 1975a). One of these isozymes, designated here as type K (for kidney), is the only pyruvate kinase found in the early fetus.[1] It is also found in significant amounts in all adult organs and tissues examined except striated muscle. A second major isozyme, type M, is found mostly in striated muscle and brain. A third isozyme, type L, is found in mammalian liver, kidney, intestines (Osterman and Fritz, 1974), and probably erythrocytes (Bigley et al., 1968; Cardenas et al., 1975a; Nakashima, 1974), though this latter assignment has been controversial (Susor and Rutter, 1971; Imamura and Tanaka, 1972; Whittell et al., 1973).

Imamura and Tanaka (1972) have suggested that type K pyruvate kinase should be regarded as the prototype and types M and L as specialized forms, since quantities of types M and L increase during differentiation of the tissues in which they are found. The data presented in this paper suggest that only one of these two specialized isozymes is present in birds, for we find pyruvate kinase isozymes in chicken tissues corresponding in distribution, and in kinetic, immunological, and electrophoretic properties only to the mammalian type K and type M isozymes. We find no evidence for a specialized isozyme in kidney, liver, intestines, or erythrocytes of chicken that would correspond to the mammalian type L pyruvate kinase.

Materials and Methods

Chickens. Adult male white leghorn chickens and fertilized white leghorn chicken eggs were obtained locally. The eggs were incubated at 38°.

Reagents. Sucrose was enzyme grade from Schwarz/Mann; except for common chemicals, all other reagents, including lactate dehydrogenase, were obtained from Sigma. Distilled deionized water was used for making all solutions.

Preparation of Tissue Extracts. Adult chickens were bled to death by heart puncture, and the blood was collected in heparinized tubes. 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 from the breast. The entire muscle mass from breast or thigh was dissected from embryos. All extractions and centrifugations were performed at 0–4°.

Tissues other than erythrocytes were extracted with a buffer consisting of 0.02 *M* Tris-HCl (pH 7.5 at room temperature), 0.15 *M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, and 0.01 *M* β-mercaptoethanol. (The mercaptoethanol was added to the extraction buffer on the day of use.) Known weights of adult skeletal muscle samples 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 volume of the buffer in a Potter-Elvehjem homogenizer. For electrophoresis, the homogenates were centrifuged at 10,000*g* for 20 min, then diluted to give solutions with 4–10 pyruvate kinase units (micromoles per min)/ml. The usual dilutions were: liver, lungs, and fetal tissues, no dilution; heart and brain, twofold; kidney and spleen, threefold; and adult skeletal muscle, tenfold.

Red cells were collected from the blood by centrifugation at 3000*g* for 10 min and then washed five times by suspending the cells in Earle's basic saline solution (Earle, 1943) and recentrifuging. The erythrocytes were then lysed by adding an equal volume of 1 m*M* EDTA and 10 m*M* β-

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[1] The nomenclature used here does not correspond to IUPAC-IUB recommendations. With at least nine naturally occurring isozymes or hybrid isozymes, and a total of 12 if in vitro hybridization is included, the recommended numerical designations become, in our opinion, far too confusing. The K, L, M nomenclature we have adopted has been widely used and is particularly convenient in naming hybrid species. However, the type K isozyme has also been referred to as type A (for adipose), M_2, PK-III, and C; the type M isozyme has been variously designated as M_1, A, and PK-II; the L (liver) isozyme is also called type B or PK-I.

mercaptoethanol to the final pellet. After lysis, red cell ghosts were removed by centrifugation at 3000*g* for 10 min.

Cellulose Acetate Electrophoresis. Electrophoretic analysis was performed on all samples at least once before freezing, but many of the electrophoretic patterns shown in this paper were obtained using frozen extracts. No differences between fresh and frozen extracts were observed.

Electrophoresis was carried out on cellulose acetate strips (1 × 6 in. Sepraphore III, Gelman Instrument Co.) at 250 V (17 V/cm) and 4° as outlined by Susor and Rutter (1971). The standard electrophoresis buffer, used unless otherwise stated, consisted of 0.02 *M* Tris-HCl (pH 8.0 at 4°), 0.5 *M* sucrose, 4 m*M* $MgCl_2$, 1 m*M* Fru-1-6-P_2, and 0.01 *M* β-mercaptoethanol.[2] Tissue extracts were dialyzed against the electrophoresis buffer for 2 hr before sample application. Replicate applications were used for low-activity extracts. Bands of pyruvate kinase were visualized after electrophoresis by the procedure of Susor and Rutter (1971), as modified by Cardenas and Dyson (1973).

Kinetic Measurements. Standard assays of pyruvate kinase activity were performed as described in the accompanying paper (Cardenas et al., 1975b). For most kinetic studies, the tissue extracts (of adult liver or of the thigh muscles of 11-day embryos) were first centrifuged at 100,000*g* for 30 min and then either (a) passed through a 1.5 × 3.0 cm column of Sephadex G-25 equilibrated with 0.05 *M* imidazole-HCl (pH 7.5), 0.10 *M* KCl, 0.01 *M* $MgCl_2$, and 0.01 *M* β-mercaptoethanol, or (b) dialyzed against the same buffer for 16 hr. Extracts prepared by the two procedures gave the same results. For one kinetic study, the enzyme was precipitated by adding ammonium sulfate to 90% of saturation. The precipitate was collected by centrifugation, washed with 90% saturated ammonium sulfate, and dissolved in and dialyzed against the G-25 column buffer for 2 hr. For another kinetic study, an extract of 11-day fetal thigh muscle was put through the G-25 column as before, then incubated at 37° for 30 min in the presence of 33 m*M* EDTA in order to determine whether two interconvertible forms exist of the type described in rats by Pogson (1968a,b) and by Walker and Potter (1973).

Kinetic studies utilized the assay medium described elsewhere (Cardenas et al., 1975) except that the pH was 7.5. When P-enolpyruvate was the variable substrate, ADP concentration was 1.0 m*M;* when ADP was the variable substrate, the P-enolpyruvate concentration was 0.2 m*M*. Fru-1,6-P_2, when present, was 1.0 m*M*. Assays were initiated by adding an aliquot of tissue extract.

Protein concentrations in the extracts were determined by the method of Folin–Ciocalteau, as described by Clark (1964).

Using the coupled assay, adult liver extracts had appreciable activity when Fru-1,6-P_2 was added without P-enolpyruvate, due apparently to utilization of Fru-1,6-P_2 by other glycolytic enzymes in the extracts. Therefore, controls containing all components except P-enolpyruvate were subtracted point-by-point from the liver assays.

The activity of enolase (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), 0.1 *M* KCl, 0.01 *M* $MgCl_2$, and varying concentrations of P-enolpyruvate. The rate of disappearance of P-enolpyruvate was calculated using an extinction coefficient of 1400 M^{-1} cm^{-1} (see Wold and Ballou, 1957).

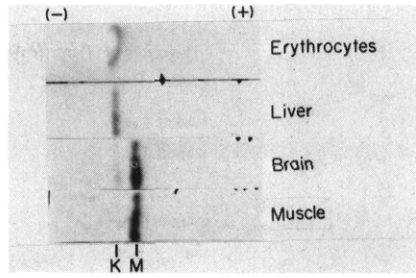

FIGURE 1: Representative pyruvate kinase zymograms of adult chicken tissues after 16 hr of electrophoresis. The centrally located dark triangles mark the origins; other notches were used for identification of samples. The zymograms in this and subsequent figures were aligned using adult brain as a standard, as explained in the text.

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 were determined by extrapolating plots of $1/v$ vs $1/s^2$ to infinite substrate; these values for maximal velocity were used in the Hill plots.

Immunological Studies. Testing of immunological cross-reactivity was done by titrating constant amounts of chicken tissue extracts with increasing quantities of antiserum made against bovine or chicken type M pyruvate kinase, prepared in rabbits as described previously (Cardenas et al., 1973). These tests were carried out in a solution consisting of 0.05 *M* potassium phosphate (pH 7.0), 0.8% NaCl, and 2 m*M* β-mercaptoethanol. The solutions were incubated at 37° for 1 hr, then at 4° for 5 hr, and centrifuged 30 min at 4000g before assaying the supernatant for the remaining pyruvate kinase activity.

Results

Figures 1–3 contain representative photographs of the 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 Figures 1–3 have been aligned with the adult brain zymogram rather than according to electrophoretic origin.

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 2–4 hr in order to detect any rapidly migrating or unstable bands of pyruvate kinase. However, in the absence of $MgCl_2$, an additional but minor rapidly migrating cathodic band was sometimes seen in zymograms of muscle extracts. We are inclined to interpret this band as an electrophoretic variant of the muscle enzyme, analogous to the minor peak seen in isoelectric focusing of purified chicken muscle pyruvate kinase (Cardenas et al., 1975b). (We note that several isoelectric variants of crystalline rabbit muscle pyruvate kinase have been reported by Susor et al. (1969).)

Figure 1 shows the distribution of the two isozymes of pyruvate kinase in typical adult tissues, while Figure 2 presents comparative electrophoresis of extracts of selected adult and embryonic tissues. Since one of the isozymes was

[2] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; Fru-1,6-P_2, fructose 1,6-bisphosphate.

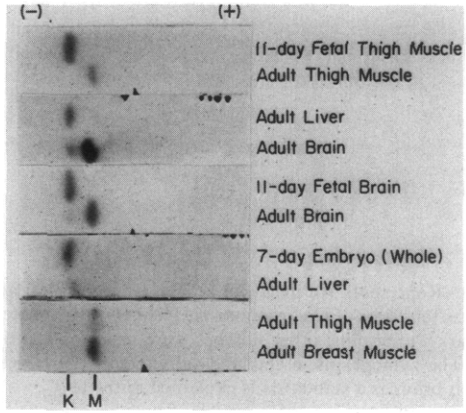

FIGURE 2: Pyruvate kinase zymograms of various adult and fetal tissues run two per cellulose acetate strip.

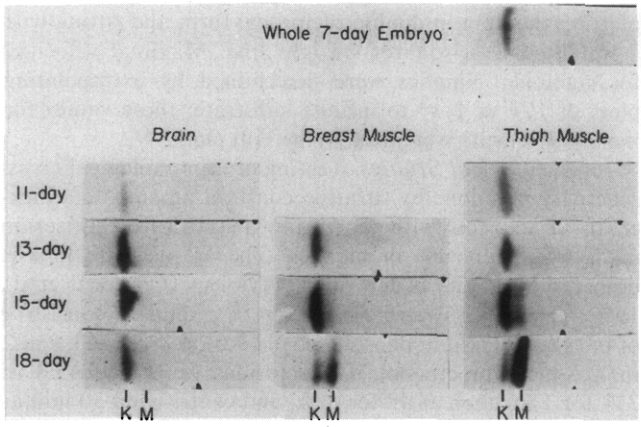

FIGURE 3: Zymograms of muscle and brain in the developing chick embryo. The time after start of incubation is given in days. Hatching occurs at 20–21 days.

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, we find 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 Figure 3 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 the adult tissues; the adult ratio of these isozymes is achieved after hatching, which occurs at 20–21 days.

Liver and kidney contain only one isozyme (type K), with no evidence of an anodic band such as the type L band seen in mammalian liver, kidney, intestines, and erythrocytes. To

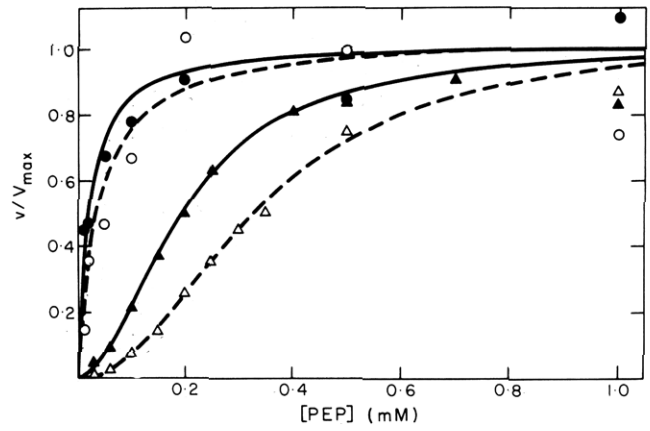

FIGURE 4: Velocity of pyruvate kinase from adult chicken liver (- - -) and from thigh muscle of 11-day fetal chick (—) as a function of phosphoenolpyruvate (PEP) concentration. The velocities are expressed as the ratio of observed velocity to maximal velocity. (Δ) Adult liver extract in the absence of Fru-1,6-P_2; (○) adult liver extract in the presence of 1.0 m*M* Fru-1,6-P_2; (▲) fetal thigh muscle extract in the absence of Fru-1,6-P_2; (●) fetal thigh muscle extract in the presence of 1.0 m*M* Fru-1,6-P_2.

minimize the possibility that there were additional bands of pyruvate kinase in chicken liver which were not separated by our regular electrophoresis buffer, extracts of liver were electrophoresed in five different buffers and for varying periods of time. The buffers consisted of (1) the standard buffer, (2) 0.05 *M* glycine-KOH (pH 10.0), (3) 0.02 *M* potassium phosphate (pH 7.0), (4) standard buffer without Fru-1,6-P_2, and (5) 0.02 *M* Tris-maleate (Ph 5.5). All buffers contained 0.5 *M* sucrose and 0.01 *M* β-mercaptoethanol. We were never able to detect more than one band of pyruvate kinase activity with any of these conditions.

Figure 4 illustrates the kinetic properties of pyruvate kinase activity from adult liver and fetal thigh muscle, determined as a function of P-enolpyruvate concentration in the absence of Fru-1,6-P_2 (triangles in Figure 4). The sigmoidal velocity curves of these extracts become hyperbolic when Fru-1,6-P_2 is added (circles). Pyruvate kinase from adult breast muscle, on the other hand, has hyperbolic kinetics that are unaffected by Fru-1,6-P_2 (Cardenas et al., 1975b). The constants calculated from the kinetic curves are listed in Table I. The Michaelis constants and conclusions concerning Fru-1,6-P_2 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 of 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 m*M*. Although these high quantities of enolase in the liver extracts would complicate the assays for pyruvate kinase by lowering the concentration of P-enolpyruvate, we calculate that the error would be no more than 10% at the highest concentrations of P-enolpyruvate used here and cannot account for the differences we see between extracts of fetal thigh muscle and adult liver.

In the accompanying paper, it is shown that rabbit antibodies produced against bovine skeletal muscle pyruvate ki-

Table I: Kinetic Constants for Pyruvate Kinase Activity of Chicken Tissues.[a]

Parameter	Extract of Adult Liver	Skeletal Muscle: 11-day Fetal Thigh	Skeletal Muscle: Adult Pectoralis
Hill coefficient	2.1	1.9	1.0
$s_{0.5}$ for P-enol-pyruvate (mM)	0.33	0.20	0.04
K_m for P-enol-pyruvate in the presence of Fru-1,-6-P_2 (mM)	0.04	0.02	0.04

[a] 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.

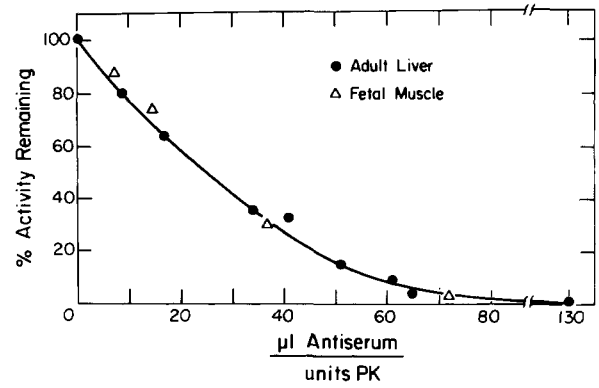

FIGURE 5: Pyruvate kinase activity remaining in adult chicken liver (●) and fetal chick thigh muscle (△) extracts after treatment with rabbit antiserum prepared by inoculation with bovine pyruvate kinase type M. The methods employed are described in the text.

nase neutralize chicken skeletal muscle pyruvate kinase (Cardenas et al., 1975b). In Figure 5 we demonstrate 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 M pyruvate kinases are immunologically similar, consistent with the report of Imamura et al. (1972) that mammalian type K and M pyruvate kinases are immunologically cross-reactive but distinct from the mammalian type L.

Discussion

Two major, noninterconvertible bands of pyruvate kinase activity were detected after electrophoresis of a variety of fetal and adult chicken tissues (Figures 1–3). Chicken type M pyruvate kinase, like its mammalian counterpart, has been found only in muscle and brain.

Chicken type K pyruvate kinase also has a pattern of tissue distribution similar to that of its mammalian counterpart. Type K is the only isozyme of pyruvate kinase found in the early mammalian fetus, and it is found in significant quantities in all adult mammalian tissues examined except skeletal muscle. Similarly, chicken type K is readily seen in all adult chicken tissues except striated muscle and is the only band seen in the whole 7-day embryo. The transition from K to M in fetal chicken skeletal muscle parallels equivalent isozyme shifts in fetal rat muscle (Osterman et al., 1973).

We could find no evidence in the zymograms for a chicken pyruvate kinase corresponding to the mammalian type L isozyme. Type L pyruvate kinase is the principal isozyme in mammalian liver and is the sole isozyme of liver parenchymal cells (Van Berkel et al., 1972; Crisp and Pogson, 1972), while type K is a minor component of mammalian liver. Mammalian kidney has predominantly type K, with a small amount of type L. Jejunal mucosa has been reported to contain 3–4% of its pyruvate kinase activity as type L, the rest consisting of type K together with hybrids of type K and L (see Osterman and Fritz, 1974). Chicken liver, kidney, and jejunum, in contrast, appear from the zymograms to have only the type K isozyme. Similarly, whereas mammalian erythrocytes probably have the type L isozyme (Bigley et al., 1968; Cardenas et al., 1975a; Nakashima, 1974), chicken erythrocytes have type K.

Since we could find no electrophoretic evidence for a chicken type L isozyme, we turned to the use of immunological and kinetic studies in an attempt to detect its presence.

Mammalian types K and M pyruvate kinase are known to be antigenically similar, while the mammalian type L isozyme is immunologically distinct both in rats (Imamura et al., 1972) and beef (J. M. Cardenas, unpublished). Antiserum prepared against the mammalian (bovine) type M enzyme cross-reacted with both the K and M isozymes of chicken, and it removed all the activity from chicken liver extracts. These results suggest that no type L pyruvate kinase occurs in chicken liver, since any type L activity would be expected to be unaffected by these treatments.

The mammalian type L pyruvate kinase is also unique in being strongly inhibited by high concentrations of ADP at low levels of P-enolpyruvate. For example, an ADP concentration of 5 mM results in more than 90% inhibition of mammalian type L activity at P-enolpyruvate concentrations around 0.2 mM (Tanaka et al., 1967; Kutzbach et al., 1973; Cardenas et al., 1975a). We were unable to demonstrate, in pyruvate kinase assays of chicken liver extracts, any appreciable substrate inhibition by ADP, again implying the absence of a type L pyruvate kinase.

Mammalian types L and K pyruvate kinases have sigmoidal kinetics with P-enolpyruvate, but the Hill coefficient of the type L isozyme is usually reported to be between 2.0 and 3.0, whereas that of the purified type K isozyme is about 1.5 (Imamura et al., 1972). The type M isozyme has hyperbolic kinetics with P-enolpyruvate and a Hill coefficient of unity. 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.

We do, however, find differences in the $s_{0.5}$ for P-enolpyruvate when we compare the pyruvate kinases in crude extracts from fetal thigh and adult chicken liver. The values were 0.33 mM for adult liver and 0.20 mM for fetal thigh muscle (see Figure 4). This difference was reproduced when the extracts were prepared by any of the procedures described under 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 we had 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, nondialyzable 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, we feel that greater confidence can be placed on the electrophoretic and immunological results than on the kinetic studies. Therefore, we propose 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. This conclusion explains the observation that 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 et al., 1973), the level of hepatic pyruvate kinase activity does not change under different dietary conditions in the pigeon (Gevers, 1967) or the chicken (Pearce, 1971).

With the possible exception of erythrocytes, the isozyme content of which has been controversial, mammalian type L pyruvate kinase is found only in those tissues where gluconeogenesis is thought to be most important—i.e., in liver, kidney, and, according to a recent paper by Anderson and Rosendall (1973), jejunal mucosa. The implication is that the properties of this isozyme are especially adapted to support gluconeogenesis. For example, activation by Fru-1,6-P_2 and inhibition by ATP leads to minimal activity during gluconeogenesis and consequent prevention of a futile loop between P-enolpyruvate and pyruvate. (The loop would involve pyruvate kinase in the catabolic direction, pyruvate carboxylase and P-enolpyruvate carboxykinase in the anabolic direction—Scrutton and Utter, 1968). 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 (type L) isozyme, and that the properties of chicken type K represent a compromise between the characteristics needed for gluconeogenic and nongluconeogenic tissues.

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