Properties of Chicken Skeletal Muscle Pyruvate Kinase and a Proposal for Its Evolutionary Relationship to the Other Avian and Mammalian Isozymes†

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ABSTRACT: Pyruvate kinase (EC 2.7.1.40) was isolated and purified from chicken and turkey breast muscle with a purification procedure very similar to that used for the bovine skeletal muscle isozyme (Cardenas, J., Dyson, R., and Strandholm, J. (1973), *J. Biol. Chem. 248,* 6931). A study of the chemical and physical properties of the chicken enzyme revealed that it is a tetramer of four apparently identical subunits, closely resembling in this and most other respects the mammalian type M isozyme. The properties of these two enzymes are similar enough to permit subunits of chicken type M pyruvate kinase to combine with subunits of mammalian type L (one of the three mammalian isozymes) to form interspecies tetrameric hybrid isozymes in relative quantities that do not differ markedly from those formed when both the M and L isozymes are of mammalian origin. The similarity between the mammalian and avian type M pyruvate kinases suggests a close evolutionary relationship. Further comparisons among the three mammalian and two avian isozymes of pyruvate kinase are consistent with a common evolutionary origin, perhaps from an ancestral form of the type K isozyme, which is the only pyruvate kinase identified in mammalian and avian embryos.

Mammalian pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) is found in at least three distinct isozymes (Susor and Rutter, 1971; Imamura and Tanaka, 1972; Whittell et al., 1973). Type M is present chiefly in skeletal muscle, heart, and brain; type L is found in liver, kidney, intestines, and probably erythrocytes (Bigley et al., 1968; Cardenas et al., 1975; Ibsen and Krueger, 1973; Nakashima, 1974). The type K isozyme is the sole pyruvate kinase of fetal tissues and is present in most or all adult mammalian tissues.

Under standard assay conditions, types K and L pyruvate kinases have sigmoidal kinetics, while type M has hyperbolic kinetics. Type M pyruvate kinase appears to be immunologically similar to the type K isozyme but antiserum to either M or K does not cross-react with type L (Imamura et al., 1972). All the pyruvate kinases are normally found as tetramers of four identical or nearly identical subunits, and all have the same requirement for both a monovalent and divalent cation, Mg^{2+} and K^+ being preferred.

In fact, the mammalian isozymes are similar enough to each other that hybrid enzymes between any two can form (Susor and Rutter, 1971; Cardenas and Dyson, 1973) with essentially random redistribution of subunit types among the tetrameric molecules.

In the accompanying paper (Strandholm et al., 1975), we report the presence in chicken tissues of only two electrophoretically distinct pyruvate kinases. These enzymes appear to correspond in their tissue distribution, kinetic, and immunological properties to the mammalian types K and M. In this paper, we report the purification and characterization of chicken skeletal muscle pyruvate kinase and describe its interspecies hybridization with the mammalian type L isozyme. These studies and the results of the accompanying paper show that the mammalian and avian type M pyruvate kinases are closely related enzymes.

Experimental Section

Materials. Substrates were obtained from Sigma Chemical Co., agarose A 1.5m was from Bio-Rad, and Sephadex CM-50 was from Pharmacia. Ammonium sulfate was special enzyme grade from Schwarz/Mann; guanidine hydrochloride, Spectroscopic grade, was obtained from Heico Inc., Delaware Water Gap, Pa. All common chemicals were reagent grade. Distilled, deionized water was used for making all solutions.

Chicken and turkey breast muscle (pectoralis major) was obtained from freshly killed or commercially frozen birds without regard to strain, gender, age, or dietary history. No differences were found in the pyruvate kinase isolated from fresh or frozen meat.

Enzyme Assays. Assays for pyruvate kinase were carried out by coupling the reaction to lactate dehydrogenase as described by Bücher and Pfleiderer (1955). The standard assay contained 1 ml of 0.05 *M* imidazole-HCl (pH 7.0), 0.10 *M* KCl, 10 m*M* $MgCl_2$, 1 m*M* phosphoenolpyruvate, 2 m*M* ADP, 0.16 m*M* NADH, and approximately 20 units (μmol/min) of lactate dehydrogenase. Temperature was controlled at 25°. One unit of pyruvate kinase activity is defined as 1 μmol of phosphoenolpyruvate converted to pyruvate per min.

Kinetic constants were determined from least-squares fits to double reciprocal plots, weighting the values proportional to v, which is equivalent to assuming a constant percent error in each velocity. Computations were carried out with an interactive program, written in Fortran and designed for a time-shared computer.

Enzyme Purification. Protein concentrations in the crude fractions were determined by the method of Folin–Ciocalteau, as described by Clark (1964). In the more purified samples, protein concentrations were estimated from the

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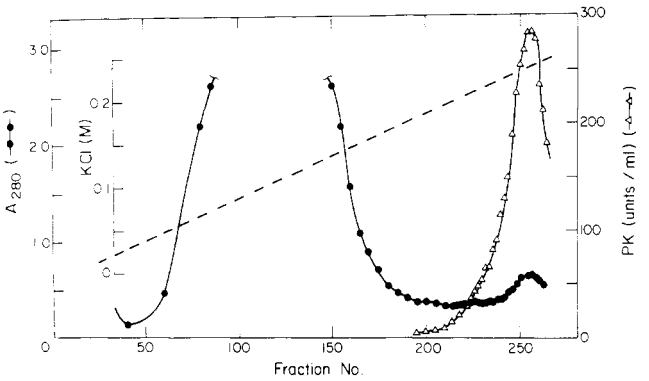

FIGURE 1: Elution of chicken muscle pyruvate kinase (PK) from carboxymethyl Sephadex by a KCl gradient. Note the large peak of contaminating proteins appearing ahead of the pyruvate kinase activity.

Table I: Purification of Pyruvate Kinase from Chicken Breast Muscle.

Fraction	Volume (ml)	Total Activity (units at 25°)	Total Protein (mg)	Specific Activity (units/mg at 25°)	Yield (percent)
Extract from 530 g	960	148,700	24,700	6.0	100
Ammonium sulfate cut	74	104,000	10,060	10.4	70
Chromatography on CM-Sephadex	177	63,600	346	184	43
Gel filtration[a]				236	

[a] Portion of sample only.

absorbance at 280 nm, using an absorptivity ($A_{280}(0.1\%)$) of 0.57 determined in this study. All steps were carried out at 0–4° unless otherwise indicated.

Extraction and ammonium sulfate fractionation were performed as described by Cardenas et al. (1973) for bovine skeletal muscle pyruvate kinase. The heat step was not used, since the enzyme from chicken breast muscle was found to be unstable at elevated temperatures.

Carboxymethyl-Sephadex type C-50 was prepared as described by Cardenas et al. (1973). Chromatography was carried out in a 5 × 40 cm column at a constant flow rate of approximately 60 ml/hr, controlled by a peristaltic pump. Up to 10 g of protein from the 67.5% ammonium sulfate precipitate was dialyzed overnight vs. the phosphate buffer and then applied to the column. After washing the column with approximately 75 ml of the phosphate buffer, a linear gradient formed from 1800 ml of the phosphate buffer and 1800 ml of 0.3 *M* KCl in the phosphate buffer (pH 6.0) was applied. A typical elution profile is shown in Figure 1. The pyruvate kinase was eluted at a KCl concentration of approximately 0.2 *M*. Fractions having the highest specific activities, generally greater than 200 units/mg of protein, were pooled and dialyzed vs. saturated ammonium sulfate and 0.01 *M* β-mercaptoethanol (pH 7.0) and stored at 0–4°

Trace contaminants remaining after carboxymethyl-Sephadex chromatography were removed from the pyruvate kinase preparation by gel filtration on Bio-Gel A-1.5m in 0.05 *M* phosphate buffer (pH 7.0), or by careful fractionation with ammonium sulfate. The enzyme was stored at 0–4° as a suspension in 80% saturated ammonium sulfate containing 10 m*M* β-mercaptoethanol. It is stable under these conditions for several months, at least.

Physical and Chemical Studies

Amino Acid Analyses. Samples of 0.2–0.3 mg were dialyzed overnight against 0.02 *M* potassium phosphate buffer and 10 m*M* β-mercaptoethanol (pH 7.0), then centrifuged at 10,000*g* for 10 min. Hydrolysis times were 21, 44, and 72 hr. Otherwise, the procedures were the same as those described earlier (Cardenas et al., 1973).

Ultracentrifuge Studies. The descriptions given earlier (Cardenas et al., 1973) were followed, except that both schlieren and interference optics were used for sedimentation velocity studies, which were carried out only at 20.0° Sedimentation coefficients were determined from schlieren images by plotting the movement of the refractive index gradient maximum. With interference optics, the second-moment method was used (Schachman, 1959; Chervenka, 1970). Sedimentation equilibrium was achieved at 14,000 rpm in 0.05 *M* potassium phosphate buffer (pH 7.1) and at 32,000 rpm in 4.07 *M* guanidine-HCl. A reducing reagent (0.01 *M* β-mercaptoethanol or 0.01 *M* dithiothreitol) was used in each case. Partial specific volumes were estimated from the amino acid analysis (Cohn and Edsall, 1943).

Absorptivity. The absorptivity was estimated by establishing the A_{280} of a sample cleared by centrifugation at 20,000*g* for 20 min, then dividing by the concentration determined in one of two ways: (1) from the amino acid analyzer, after correction for destruction of labile amino acids; or (2) by a fringe count from a synthetic boundary experiment in the analytical ultracentrifuge, using interference optics and assuming 3.88 fringes per mg per ml (Cardenas et al., 1973).

Gel Electrophoresis and Isoelectric Focusing. The procedure for isoelectric focusing was that recommended by LKB Instruments, Inc., using the apparatus and ampholines (pH 7–10) sold by them. Disc gel electrophoresis at pH 9.5 followed the procedure summarized by Gabriel (1971).

Immunological Studies. Antiserum was prepared as described previously (Cardenas et al., 1973). Immunological tests were performed using the quantitative precipitation technique similar to that described by Cardenas and Wold (1971), measuring pyruvate kinase activity remaining in the supernatant after treatment with antisera and centrifugation to remove precipitated protein.

Enzyme Hybridization and Detection. Details of the procedure were described by Cardenas and Dyson (1973). Hybridization was accomplished by denaturation in 4 *M* guanidine hydrochloride followed by dilution into 0.5 *M* sucrose, 5 m*M* dithiothreitol, 0.05 *M* Tris-HCl, 0.1 *M* HCl, and 5 m*M* $MgCl_2$ (pH 7.5). Electrophoresis of the renatured enzymes on cellulose acetate (Sepraphore III, Gelman Inst. Co.) and subsequent activity detection were performed as previously described (Susor and Rutter, 1971; Cardenas and Dyson, 1973). Images were recorded on Kodak spectrum analysis plates, No. 1.

Heat Stability. The purified enzymes, at a protein concentration of 0.33 mg/ml, were dialyzed vs. 0.05 *M* imidazole-HCl and 10 m*M* β-mercaptoethanol (pH 7.0) at 25°. Aliquots of 0.4 ml were incubated for 5 min at the indicated temperature. After incubation, the tubes were placed in an ice–water bath before assaying 30–60 min later.

Results

A slightly modified version of the purification procedure

Table II: Amino Acid Analyses of Skeletal Muscle Pyruvate Kinases.

Amino Acid	Chicken[a]	Bovine[b]	Rabbit[c]
Lysine	148 ± 9	150	148
Histidine	72 ± 4	60	62
Ammonia	219 ± 55	228	167
Arginine	125 ± 8	133	113
Cysteic acid	33	39	36
Aspartic acid and asparagine	190 ± 5	194	202
Threonine	103 ± 6	109	104
Serine	97 ± 4	126	120
Glutamic acid and Glutamine	188 ± 6	200	206
Proline	81 ± 5	89	92
Glycine	162 ± 4	162	167
Alanine	219 ± 4	219	244
Valine	166 ± 4	174	185
Methionine	70 ± 8	69	71
Isoleucine	126 ± 5	134	135
Leucine	141 ± 4	160	163
Tyrosine	28 ± 5	35	38
Phenylalanine	63 ± 1	65	65
Tryptophan	15 ± 1	12	12

[a] Residues per 212,000 g, from this study. The standard deviation is given for a total of seven analyses on two different enzyme isolations. [b] Residues per 230,000 g, from Cardenas et al., 1973. [c] Residues per 237,000 g, from Cottam et al., 1969.

used for bovine skeletal muscle pyruvate kinase (Cardenas et al., 1973) was found to be applicable to the isolation of pyruvate kinase from chicken and turkey breast muscle (pectoralis major). Purification of the chicken muscle isozyme is summarized in Table I. Similar results were obtained for the skeletal muscle isozyme of turkey. The only major difference in the isolation procedures of fowl and mammalian skeletal muscle pyruvate kinase is elimination of the heat step, as we find the enzyme from fowl to be much less stable at elevated temperatures than its mammalian counterpart. For example, after 5 min at 55°, bovine skeletal muscle pyruvate kinase retained 48% of its activity, the chicken breast muscle enzyme only 0.26%.

Sedimentation equilibrium experiments with chicken breast muscle pyruvate kinase produced linear ln c vs. r^2 plots both in dilute phosphate buffer and in a dissociating agent (guanidine-HCl). These results suggest homogeneity in mass. A partial specific volume of 0.74 ml/g, calculated from the amino acid profile in Table II, was used in all calculations. Sedimentation equilibrium in dilute phosphate buffer yielded a value of 212,000 for the molecular weight of the native enzyme, whereas sedimentation equilibrium in a dissociating medium (4 M guanidine hydrochloride) gave a value of about 53,000, indicating the presence of four subunits. The observed range in both cases was about ±3000 in a total of nine experiments. Sedimentation equilibrium of the turkey enzyme revealed that it also is a tetramer, although the molecular weight seemed to be a few percent higher than that of the chicken.

The sedimentation pattern obtained with purified chicken muscle pyruvate kinase is shown in Figure 2. The symmetry of the boundary is consistent with the presence of a single sedimenting species. A least-squares determination of the concentration dependence of sedimentation, using the assumption that $s_{20,w} = s_{20,w}(1 - kc)$, yields an $s_{20,w}^{\circ} = 9.58 \pm 0.04$ S, with a k of 8×10^{-3} ml/mg.

When concentrations were estimated from amino acid analyses, an absorptivity ($A_{280}(0.1\%)$) of 0.56 per cm path

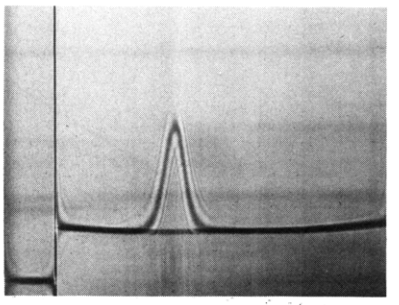

FIGURE 2: Sedimentation of chicken muscle pyruvate kinase about 45 min after reaching 56,000 rpm at 5.5°. Phaseplate angle was 70°.

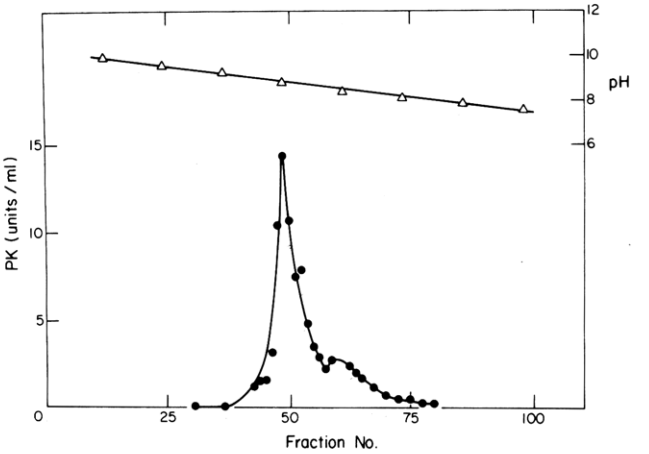

FIGURE 3: Isoelectric focusing of purified chicken muscle pyruvate kinase (PK). See Methods for details.

length was obtained with the chicken enzyme. When the concentration was determined from the analytical ultracentrifuge, using its interference optics as a differential refractometer, an absorptivity of 0.57 was obtained. The two values agree within experimental error. The value of 0.57 was used in subsequent calculations.

The isoelectric focusing profile obtained with chicken breast muscle pyruvate kinase is shown in Figure 3. An isoelectric point of 8.77 was obtained, with a minor peak of pyruvate kinase activity at pH 8.45. While slight microheterogeneity in isoelectric pH apparently does occur in our preparations of chicken breast muscle pyruvate kinase, it is much less extensive than that seen by Susor et al. (1969) in commercial samples of rabbit skeletal muscle pyruvate kinase.

The kinetic behavior of the chicken enzyme when substrates are varied can be seen in Figure 4, with the results summarized in Table III. Addition of 1 mM fructose 1,6-bisphosphate did not significantly affect the kinetics of the enzyme. The apparent K_m for K^+ was 11 mM and for Mg^{2+} 0.5 mM, both at 2 mM ADP and 1 mM phosphoenolpyruvate.

When the enzymatic activity of chicken muscle pyruvate kinase was titrated with antiserum prepared against the bovine muscle enzyme, complete neutralization of the chicken enzyme was achieved at approximately 3–4 times the quantity of antiserum needed to completely neutralize the bovine enzyme. The two enzymes are therefore immunologically very similar but not identical.

Figure 5 compares the electrophoretic mobility of bovine and chicken type M pyruvate kinases in polyacrylamide. Other comparisons between the two type M enzymes are

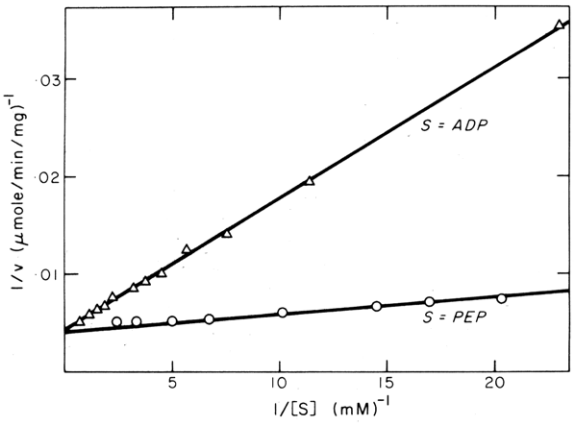

FIGURE 4: Kinetic behavior of purified chicken muscle pyruvate kinase when phosphoenolpyruvate (PEP) is varied at 1.32 m*M* ADP, and when ADP is varied at 0.368 m*M* phosphoenolpyruvate. See Table III for constants. The extrapolated maximum velocity was 250 units/mg in each case.

Table III: Comparative Properties of Bovine and Chicken Skeletal Muscle Pyruvate Kinases.

Parameter	Chicken[a]	Bovine[b]
K_m for ADP	0.3 m*M*	0.4 m*M*
K_m for phosphoenolpyruvate	0.05 m*M*	0.04 m*M*
Cation requirements	Mg^{2+}, K^+	Mg^{2+}, K^+
V_{max} (μmol per min per mg at 25°)	250	400
Molecular weight	212,000	230,000
Subunit molecular weight	53,000	57,000
Number of subunits	4	4
Partial specific volume	0.74	0.74
Absorptivity A_{280} (0.1%)	0.57	0.55
A_{280}/A_{260}	1.75	1.71
Sedimentation coefficient ($s_{20,w}^0$)	9.58 S	9.94 S
Concentration dependence of $s_{20,w}$	8×10^{-3} ml/mg	8×10^{-3} ml/mg
Isoelectric pH	8.8	8.9

[a] From this study. [b] From Cardenas et al., 1973.

given in Tables II and III. Figure 6 shows an interspecies hybridization between the chicken breast muscle enzyme and the allosteric type L isozyme from bovine liver.

Discussion

The results described here and the data summarized in Tables II and III reveal that chicken skeletal muscle pyruvate kinase closely resembles a typical mammalian type M counterpart, with only a few notable differences (e.g., in heat stability). Similarly between the two enzymes is emphasized by the fact that we could isolate skeletal muscle pyruvate kinase from domestic fowl with only minor changes in the procedure used earlier for the bovine enzyme (Cardenas et al., 1973). The homogeneity of the final preparation is demonstrated in the symmetry of the refractive index gradient during sedimentation velocity (Figure 2), the linearity of the sedimentation equilibrium plot of the native enzyme and of its subunits, and by the single band found after electrophoresis in polyacrylamide (Figure 5). The small differences in isoelectric points and molecular weights are reflected in the fact that the two enzymes can be separated on disc gel electrophoresis at pH 9.5 (see Figure 5).

The similarity between mammalian and chicken muscle

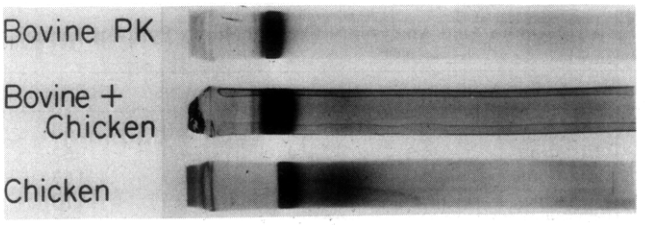

FIGURE 5: Comparative disc gel electrophoresis of type M pyruvate kinases (PK) isolated from bovine and chicken skeletal muscle.

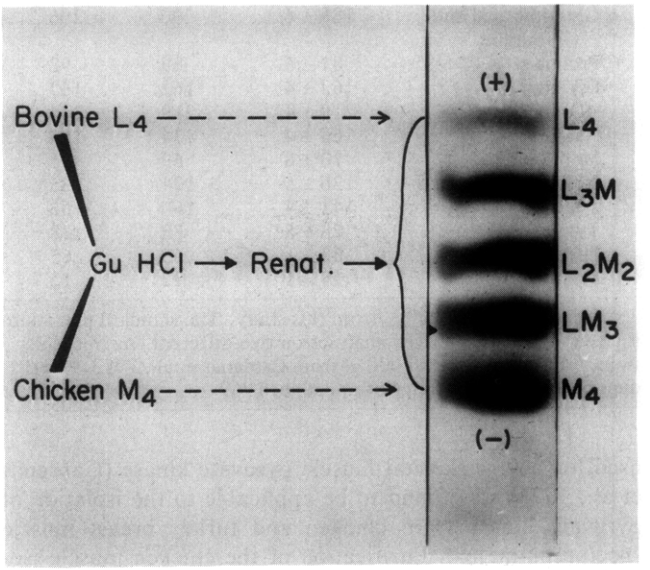

FIGURE 6: Hybridization of chicken skeletal muscle pyruvate kinase (M_4) with the allosteric (L_4) isozyme of pyruvate kinase from bovine liver. Indicated at the right of the figure are the relative numbers of type M and type L subunits for each species.

pyruvate kinases must extend to their tertiary structure, at least at regions of intersubunit contact, for we find that chicken type M pyruvate kinase, like the bovine type M isozyme, can be hybridized with the bovine liver isozyme (Figure 6).

Similar comparisons between the mammalian and avian type K isozymes are not possible, as the avian type K pyruvate kinase has not yet been purified. However, we show in the accompanying paper (Strandholm et al., 1975) that avian type K pyruvate kinase crossreacts with antiserum prepared against a mammalian type M isozyme, and that the kinetic properties of the avian and mammalian type K pyruvate kinases are similar in many respects. Thus, it appears that both of the avian isozymes of pyruvate kinase correspond rather closely to their mammalian counterparts in spite of the apparent absence in chicken tissues of an isozyme equivalent to the third mammalian form, type L.

Imamura and Tanaka (1972) have suggested that the ontogenetic relationship of the mammalian pyruvate kinases (types L and M appear only late in development) point to the type K isozyme as the more primitive species, with the M and L isozymes representing differentiated forms. Recent data from this laboratory and others support that thesis. In particular, the three mammalian isozymes have very similar molecular weights, subunit structure, and cation requirements (Imamura et al., 1972; Cardenas et al., 1973; Cardenas and Dyson, 1973; Kutzbach et al., 1973); subunits of any one of the three may form hybrid tetramers with subunits from either of the other two isozymes (Susor

and Rutter, 1971; Cardenas and Dyson, 1973); and the positive allosteric effector of the K and L isoenzymes, fructose 1,6-bisphosphate, has now been found to bind to and under certain conditions to influence the kinetics of the type M isozyme as well (Kayne and Price, 1973; Cardenas et al., 1975).

In addition, there are other kinetic similarities between the type M and type K isozymes (Imamura et al., 1972) and, as elegantly demonstrated in the pioneering study of Koler et al. (1964), strong homologies in the pattern of fragments from trypsin digestion of the type K and type L isozymes of leukocytes and erythrocytes, respectively.

That the K and L isozymes are, in fact, the products of different genes is demonstrated by the genetic studies of Koler et al. (1964) and Bigley et al. (1968). There is no direct proof at present that type M pyruvate kinase is the product of a third gene, but the reported differences in mass, kinetic, immunological, electrophoretic, and developmental properties of the three isozymes strongly point to that conclusion. It seems probable from present evidence that the L and M isozymes evolved by duplication of the type K gene to supply certain highly differentiated tissues with a pyruvate kinase having kinetic properties better suited to the special metabolic requirements of those tissues.

In particular, the type M isozyme is a very efficient catalyst for the conversion of phosphoenolpyruvate to pyruvate, is not greatly influenced by effectors of the other isozymes (notably fructose 1,6-bisphosphate), and is relatively insensitive to substrate inhibition by ADP and to product inhibition by ATP (Cardenas et al., 1974). This latter point is significant, as there is reason to believe that reversal of this enzyme (that is, phosphorylation and enolization of pyruvate) is essential to glyconeogenesis in muscle (Dyson et al., 1975). In accordance with these properties, the type M isozyme is found in tissues that depend heavily on the Embden–Meyerhof pathway for energy—e.g., in muscle and brain.

The type L isozyme of mammals, on the other hand, is very sensitive to feed-forward activation by fructose 1,6-bisphosphate and to inhibition by ATP, which are characteristics needed to prevent a "futile loop" between phosphoenolpyruvate and pyruvate when pyruvate kinase is bypassed during gluconeogenesis (Scrutton and Utter, 1968). This enzyme is found in tissues that synthesize and secrete significant quantities of free glucose—liver, kidney, and, according to a recent paper by Anderson and Rosendall (1973), small intestine.

The homologies between the mammalian and avian type M pyruvate kinases and, although with less available data, between the mammalian and avian type K enzymes, can be explained if the type M isozyme evolved prior to divergence of the mammalian and avian vertebrate classes. If that is true, one should find isozymes equivalent to the mammalian M and K in reptiles and perhaps even in more primitive vertebrates. There is kinetic evidence for the existence of at least two isozymes of pyruvate kinase in turtles (Kornecki-Gerrity and Penney, 1974a,b), and kinetic, electrophoretic, and immunological evidence for the existence of three pyruvate kinase isozymes in a frog, *Rana pipiens* (Schloen et al., 1974). In both cases, one of the isozymes has many of the properties associated with mammalian type M pyruvate kinase.

The evolutionary history of the type L isozyme of mammals is less clear. While no evidence for this isozyme could be found in the chicken (Strandholm et al., 1975), Schloen et al. (1974) found a frog liver isozyme of pyruvate kinase that is immunologically distinct from the skeletal muscle isozyme, a situation similar to that of mammals. However, in the absence of any direct comparison between the presumptive type L isozyme of frogs and the type L isozyme of mammals, it would be impossible to predict whether these isozymes are likely to have evolved in a common event. Likewise, we cannot predict whether the absence of an L isozyme in chickens is because it failed to appear in the evolutionary lines leading to birds, or whether this isozyme was for some reason lost. Either position is equally well supported by the observation that the chicken type K isozyme seems to have properties somewhat between those of the mammalian types K and L (Strandholm et al., 1975).

Further classification of the relationship between the pyruvate kinases of birds, mammals, and more primitive species must await additional direct comparisons of the isozymes in question. We hope that this discussion will encourage interested workers to undertake such an investigation.

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References

Anderson, J. W., and Rosendall, A. F. (1973), *Biochim. Biophys. Acta 304,* 384.

Bigley, R. H., Stenzel, P., Jones, R. T., Campos, J. O., and Koler, R. D. (1968), *Enzymol. Biol. Clin. 9,* 10.

Bücher, T., and Pfleiderer, G. (1955), *Methods Enzymol. 1,* 435.

Cardenas, J. M., and Dyson, R. D. (1973), *J. Biol. Chem. 248,* 6938.

Cardenas, J. M., Dyson, R. D., and Strandholm, J. J. (1973), *J. Biol. Chem. 248,* 6931.

Cardenas, J. M., Dyson, R. D., and Strandholm, J. J. (1975), in Isozymes: Molecular Structure, Markert C., Ed., New York, N.Y., Academic Press, p 523.

Cardenas, J. M., and Wold, F. (1971), *Arch. Biochem. Biophys. 144,* 663.

Chervenka, C. H. (1970), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Spinco Division of Beckman Instruments.

Clark, J. M., Jr. (1964), Experimental Biochemistry, San Francisco, Calif., W. H. Freeman, pp 109 and 219.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 370.

Cottam, G. L., Hollenberg, P. F., and Coon, M. J. (1969), *J. Biol. Chem. 244,* 1481.

Dyson, R. D., Cardenas, J. M., and Barsotti, R. J. (1975), *J. Biol. Chem.* (in press).

Gabriel, O. (1971), *Methods Enzymol. 22,* 565.

Ibsen, K. H., and Krueger, E. (1973), *Arch. Biochem. Biophys. 157,* 509.

Imamura, K., and Tanaka, T. (1972), *J. Biochem. (Tokyo) 71,* 1043.

Imamura, K., Taniuchi, K., and Tanaka, T. (1972), *J. Biochem. (Tokyo) 72,* 1001.

Kayne, F. J., and Price, N. C. (1973), *Arch. Biochem. Biophys. 159,* 292.

Koler, R. D., Bigley, R. H., Jones, R. T., Rigas, D. A., Vanbellinghen, P., and Thompson, P. (1964), *Cold Spring*

Harbor Symp. Quant. Biol. 29, 213.
Kornecki-Gerrity, E. H., and Penney, D. G. (1974a), *Comp. Biochem. Physiol. B 48,* 507.
Kornecki-Gerrity, E. H., and Penney, D. G. (1974b), *Comp. Biochem. Physiol. B 49,* 15.
Kutzbach, C., Bischofberger, H., Hess, B., and Zimmermann-Telschow, H. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1473.
Nakashima, K. (1974), *Clin. Chim. Acta 55,* 245.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N.Y., Academic Press.
Schloen, L. H., Kmioteck, E. H., and Sallach, H. J. (1974), *Arch. Biochem. Biophys. 164,* 254.
Scrutton, M. C., and Utter, M. F. (1968), *Annu. Rev. Biochem. 37,* 249.
Strandholm, J. J., Cardenas, J. M., and Dyson, R. D. (1975), *Biochemistry,* preceding paper in this issue.
Susor, W. A., Kochman, M., and Rutter, W. J. (1969), *Science 165,* 1260.
Susor, W. A., and Rutter, W. J. (1971), *Anal. Biochem. 43,* 147.
Whittell, N. M., Ng, D. O. K., Prabhakararao, K., and Holmes, R. S. (1973), *Comp. Biochem. Physiol. B, 46,* 71.

1-Halo Analogs of Dihydroxyacetone 3-Phosphate. The Effects of the Fluoro Analog on Cytosolic Glycerol-3-Phosphate Dehydrogenase and Triosephosphate Isomerase†

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ABSTRACT: 1-Fluoro-3-hydroxyacetone phosphate (fluoroacetol phosphate) has been prepared by oxidation of 1-fluoro-3-chloro-2-propanol to 1-fluoro-3-chloroacetone, phosphorylation with silver dibenzylphosphate, and the intermediate isolation of 1-fluoro-3-hydroxyacetone phosphate dibenzyl ester, followed by catalytic hydrogenation and preparation of the stable monosodium salt. The chloro analog as the pure, stable monosodium salt has been prepared by a similar route from 1,3-dichloroacetone. 1-Fluoro-3-hydroxyacetone-P is a substrate for cytosolic NAD^+-linked glycerol-3-P dehydrogenase (EC 1.1.1.8) from rabbit skeletal muscle with an apparent K_m of 50 mM under conditions in which dihydroxyacetone-P exhibits an apparent K_m of 0.15 mM. Under these conditions the fluoro analog is 85% hydrated whereas dihydroxyacetone-P has been shown by others to be 44% hydrated. The turnover numbers are 49,000 molecules of NADH oxidized per minute per molecule of enzyme at 25° with the fluoro analog as substrate, and 60,000 with dihydroxyacetone-P as substrate. The product of the reduction of the fluoro analog has been identified as 1-fluorodeoxyglycerol-3-P. 1-Fluoro-3-hydroxyacetone-P is a comparatively weak irreversible inhibitor at 4° of rabbit muscle triosephosphate isomerase (EC 5.3.1.1) with a second-order rate constant of 2.6 M^{-1} sec^{-1}. Inhibition by pyrazole in vivo of the alcohol dehydrogenase catalyzed oxidation of 1-fluorodeoxyglycerol-3-P indicates that in mice the reduction of 1-fluoro-3-hydroxyacetone-P to L-1-fluorodeoxyglycerol-3-P is not a significant metabolic route, or that an alternative route exists when the alcohol dehydrogenase dependent pathway is inhibited.

1-Fluoro analogs of glycerol-3-P and dihydroxyacetone-3-P may be able to exert differential effects on pathways to phospholipids in normal and neoplastic cells, by virtue of their potential behavior as substrates for cytosolic glycerol-3-P dehydrogenase (Fondy et al., 1970, 1974; Ghangas and Fondy, 1971) or by inhibition of the acyldihydroxyacetone-P and acylglycerol-3-P pathways to phosphatidic acid and ether lipids. These possibilities have led us to carry out the synthesis of racemic and optically pure forms of 1-fluorodeoxyglycerol-3-P in the work cited above. Independent syntheses have been carried out by Lloyd and Harrison (1971, 1973). We have also proposed and prepared secondary fluorodeoxyketohexoses as potential intracellular precursors of the fluorotrioses (Rao et al., 1975).

In this work we present the first successful synthesis of 1-fluoro-3-hydroxyacetone-P along with alternative approaches to the synthesis of the chloro and bromo analogs. The chloro, bromo, and iodo analogs of dihydroxyacetone-P (haloacetol phosphates) were originally prepared by Hartman (1968, 1970) employing a route not readily applicable to preparation of the fluoro analog. An independent synthesis of the bromo analog has also been developed by Coulson et al. (1970). A preliminary report of our work has been presented (Fondy et al., 1971).

We have examined 1-fluoro-3-hydroxyacetone-P as a substrate for cytosolic NAD^+-linked glycerol-3-P dehydrogenase from rabbit skeletal muscle, and have identified the

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