Loechler, E., and Hollocher, T. (1975), *J. Am. Chem. Soc. 97,* 3235–3237.
Main, L., Kasperek, G., and Bruice, T. (1972), *Biochemistry 11,* 3991–4000.
Massey, V., and Curti, B. (1966), *J. Biol. Chem. 241,* 3417–3423.
Massey, V., and Ganther, H. (1965), *Biochemistry 4,* 1161–1173.
Massey, V., and Gibson, Q. (1964), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23,* 18–29.
Massey, V., Palmer, G., Williams, C., Swoboda, B., and Sands, R. (1966), in Flavins and Flavoproteins, E. Slater, Ed., Amsterdam, Elsevier, 133–158.
Moore, S., and Stein, W. (1954), *J. Biol. Chem. 211,* 907–913.
O'Brien, D., Weinstock, L., and Cheng, C. (1970), *J. Heterocycl. Chem. 7,* 99–105.
Oppenheimer, N., Arnold, L., and Kaplan, N. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3200–3205.
Porter, D., Voet, J., and Bright, H. (1973), *J. Biol. Chem. 248,* 4400–4416.
Rando, R. (1973), *J. Am. Chem. Soc. 95,* 4438–4439.
Rubenstein, P., and Strominger, J. (1974), *J. Biol. Chem. 249,* 3782–3788.
Shinkai, S., and Bruice, T. (1973), *J. Am. Chem. Soc. 95,* 7526–7528.
Shinkai, S., Kunitake, T., and Bruice, T. (1974), *J. Am. Chem. Soc. 96,* 7140–7141.
Shore, J., Gutfreund, H., Brooks, R., Santiago, D., and Santiago, P. (1974), *Biochemistry 13,* 4182–4191.
Spencer, R., Fisher, J., Laura, R., and Walsh, C. (1976a) in Flavins and Flavoproteins, T. Singer, Ed., Amsterdam, Elsevier (in press).
Spencer, R., Fisher, J., and Walsh, C. (1976b), *Biochemistry,* preceding paper in this issue.
Sund, H. (1968), in Biological Oxidations, T. Singer, Ed., New York, N.Y., Wiley-Interscience, pp 621–624.
Swoboda, B. (1969), *Biochim. Biophys. Acta 175,* 365–379.
Swoboda, B., and Massey, V. (1966), in Flavins and Flavoproteins, E. Slater, Ed., Amsterdam, Elsevier, 263–282.
Walsh, C., Krodel, E., Massey, V., and Abeles, R. (1973a), *J. Biol. Chem. 248,* 1946–1955.
Walsh, C., Lockridge, O., Massey, V., and Abeles, R. (1973b), *J. Biol. Chem. 248,* 7049–7054.
Walsh, C., Schonbrunn, A., and Abeles, R. (1971), *J. Biol. Chem. 246,* 6855–6866.
Williams, R., Shinkai, S., and Bruice, T. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1763–1767.
Wu, F., McKenzie, R., and McCormick, D. (1970), *Biochemistry 9,* 2219–2224.
Yokoe, I., and Bruice, T. (1975), *J. Am. Chem. Soc. 97,* 450–451.

Electrofocusing and Kinetic Studies of Adult and Embryonic Chicken Pyruvate Kinases†

Kenneth H. Ibsen,* Lowell Murray,‡ and Steven W. Marles

ABSTRACT: Chicken embryos less than 15 days old contain only the K isozyme of pyruvate kinase, which appears to exist in vivo as an R,T conformational set with pI values of 7.2 and 6.6, respectively. Sets of lower pI and higher pI K-isozyme variants also are obtained. Whole embryos of 15 days or more of development show progressively increasing amounts of higher pI, lower $K_{0.5S}$ enzymatic variants. Tissue distribution and kinetic properties suggest that the highest pI form (pH 8.8–9.0) is an M-isozyme analogue. The intermediate forms are postulated to be hybrids. Adult liver extracts contain only the embryonic K isozyme; no evidence for an L-isozyme analogue was obtained. All major forms of the enzymes are compared with respect to saturation by phosphoenolpyruvate in the absence of effector and in the presence of fructose 1,6-diphosphate, alanine, serine, phenylalanine, tryptophan, and/or Mg-ATP.

Rat tissues contain three basic noninterconvertible pyruvate kinases. They are: the K form[1], regarded by Imamura and Tanaka (1972) as the prototype enzyme since it is the fetal enzyme as well as the major isozyme of many normal adult tissues; the L form, found as the major liver enzyme and as a minor kidney cortex enzyme; and the M form, found in differentiated skeletal muscle, brain, and heart. Hybrids may exist in the erythrocyte (Imamura and Tanaka, 1972; Peterson et al., 1974) and kidney (Cardenas et al., 1975b).

Surveys of organs from many other mammals show all to have similar isozyme patterns (Schulz and Sparmann, 1972; Imamura and Tanaka, 1972; Whittell et al., 1973; Balinsky et al., 1973; Carbonell et al., 1973; Osterman and Fritz, 1973; Imamura et al., 1973; Kozhevnikovn, : 1973; Ibsen et al., 1975a; Cardenas et al., 1975b). Thus among mammals, homology may be assumed.

The mammalian L and M isozymes appear to be homotetramers (Kayne, 1973; Cardenas et al., 1973; Cardenas and Dyson, 1973). Hybridization studies show the bovine K isozyme also is likely to be a homotetramer (Cardenas et al., 1975b). Therefore, unless the one isozyme arises from an-

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[1]The K, L, and M nomenclature for pyruvate kinase isozymes is employed in this study. Various nomenclatures have been compared (Osterman et al., 1973; Yanagi et al., 1974; Schloen et al., 1974; Strandholm et al., 1975; Ibsen et al., 1975a).

other by epigenetic modification, as once suggested (Ibsen and Trippet, 1972), the three isozymes should be coded for by distinct genes. The generally independent tissue distribution (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Whittell et al., 1973; Farina et al., 1974; Cardenas et al., 1975b) and the nonreciprocal regulation of the three isozymes (Krebs and Eggleston, 1965; Tanaka et al., 1967, 1972; Suda et al., 1972; Nakamura et al., 1972; Ibsen et al., 1975a) suggest one is not formed from another. This leaves the three-gene hypothesis as the most plausible one.

Organisms outside of the mammalian family also have multimolecular forms of pyruvate kinase. These include: *E. coli* (Malcovati et al., 1973); *Mucor rouxii* (Friedenthal et al., 1973); locust (Bailey and Walker, 1969); oyster (Mustafa and Hochachka, 1971); trout (Somero and Hochachka, 1968); *Rana pipiens* (Schloen et al., 1974); *Xenopus laevis* (Ibsen et al., to be published); turtles (Kornecki-Gerrity and Penny, 1974); quail shell gland (Yamada, 1974); pigeon (Gabrielli and Baldi, 1972); and chickens (Cardenas et al., 1975a,b; Strandholm et al., 1975).

Reported herein is an electrofocusing investigation of the isozyme patterns of chicken organ extracts. Despite the fact that some ten different bands of activity were obtained, it is concluded, in agreement with Cardenas et al. (1975b) and Strandholm et al. (1975), that chicken tissues contain homologues of mammalian K and M isozymes but no L isozyme. Of the ten bands isolated, six appear to be K-isozyme variants, one the M_4 homotetramer, and three KM hybrids. The K and M isozymes bear remarkable similarity to their mammalian counterparts. An attempt is made to relate the six K variants to forms previously described.

Materials and Methods

Chickens. All data reported were obtained using white Leghorn cocks or fertilized eggs obtained locally. Eggs were incubated at 39 °C. Similar data were obtained using Rhode Island red-white Leghorn hybrids.

Reagents. All biochemical reagents were of the highest grade available from Sigma or Calbiochem. Common chemicals were all analytical grade or the equivalent. Ampholines were obtained from LKB.

Preparation of Tissue Extracts. Adult cocks were killed by an intravenous overdose of phenobarbitol. Embryos and young chicks were decapitated. Tissue extracts and electrofocusing studies were performed as described (Ibsen and Trippet, 1972), except in most studies 1% pH 3.5 to 10 ampholine gradients were used. Electrofocusing in the presence of ^{14}C labeled fru-1,6-P_2[2] was done as described by Ibsen et al. (1975b), except that fresh extracts were employed and pre-incubation was eliminated. Recombination studies were performed as described by Cardenas et al. (1973), except that guanidine-HCl was removed by dialysis against a solution containing either 50 mM triethanolamine, pH 7.0, 5 mM $MgSO_4$, 15 mM KOH, 50 mM KCl, 10% glycerol or containing 10 mM KH_2PO_4 adjusted to pH 7.0 with 6 mM KOH, 60 mM KCl, 5 mM $MgSO_4$, 10% glycerol, and 12 mM β-mercaptoethanol. The latter solution promoted greater recovery of activity.

Kinetic Measurements. Kinetic assays were conducted at pH 7.5 using conditions described by Ibsen and Trippet (1973), except the enzyme was not dialyzed after isoelectrofocusing. Some preparations were stored at −70 °C after focusing and prior to kinetic analysis. In the absence of ADP but in the presence of P-enolpyruvate and/or fru-1,6-P_2, the rate of NADH oxidation was minimal, indicating the absence of relevant contaminating enzymes.

Kinetic constants for hyperbolic curves were obtained using the Lineweaver-Burk double reciprocal plot. Kinetic constants for data yielding sigmoidal rate plots were determined using the Hill plot.

[2]Abbreviations: P-enolpyruvate, phosphoenolpyruvate; fru-1,6-P_2, fructose 1,6-diphosphate.

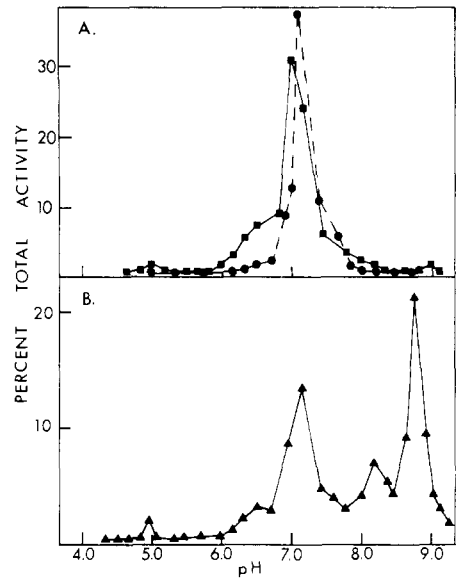

FIGURE 1. Typical pyruvate kinase activity profiles obtained when whole embryonic extracts were electrofocused. Panel A shows data obtained from an extract of an embryo which had developed for 8 ● and 15 ■ days. Panel B shows data obtained from an extract of an embryo obtained just prior to hatching after 21 days of development.

Table I: Relative Proportion of the Major Pyruvate Kinase Variants during Embryonic Development.

Days after Fertilization	Percent of Total Activity at pH		
	6.6–7.2	8.1	8.8–9.0
8	99.0		
11	99.0		0.5
15	98.5	0.6	0.9
17	95.3	0.5	4.2
19	62.3	8.0	14.8
21	39.8	12.5	34.3

Results

The Developing Embryo. At least two extracts prepared from whole chick embryos at 8, 11, 15, 17, and 21 days of development were electrofocused. As a rule, embryos 15 days or younger showed more than 90% of the total activitv at pH 7.1–7.2 (Figure 1). However, in an 11 day embryo, the predominant activity peak was obtained at pH 6.6. Other preparations commonly yielded a minor peak at this pH (Figure 1). Traces of activity also were obtained at about pH 4.5 or 5.0 (Figure 1). In addition, one of three electrofocused 8 day extracts differed from the younger embryos in that it had two additional peaks of activity at pH 8.2 and 8.6. This preparation yielded about 5% of the total activity in a band which peaked at pH 4.6, 54% in a band peaking at pH 7.1, 10% at pH 8.2, and 30% at pH 8.6.

Figure 1 illustrates and Table I quantitates the increasing proportion of activity which occurs at about pH 8.1 and 8.9

Table II: Characteristics of Pyruvate Kinases Obtained from Embryonic Extracts.

Characteristic	Variants Found in 8 to 21 Day Embryonic Extracts			Variants Found Only after 15 Days of Development		Derived or Uncommon Forms		
Mean pH	4.42	5.07	6.64	7.18	8.14	8.86	8.2[a]	8.6[a]	8.8[b]
pI value ± SE (n)	±0.05 (3)	±0.05 (3)	±0.08 (4)	±0.02 (8)	±0.06 (4)	±0.07 (4)	(1)	(1)	(1)
$K_{0.5S}$ value, mM P-enolpyruvate									
No effector				0.12 (1.4)[c]	0.024 (1.0)	0.020 (1.0)	0.27 (2.2)	0.18 (1.9)	0.17 (1.2)
5 mM Ala[a]				0.21 (1.8)	0.026 (1.0)	0.020 (1.0)			
5 mM Phe				0.70 (1.2)	0.071 (>1.0)	0.059 (1.0)			
5 mM Ser				0.060 (1.2)					
5×10^{-5} M fru-1,6-P_2				0.022 (0.9)					

[a] Found in one 8 day embryo extract. [b] Derived from pH 7.2 enzyme by incubation with fru-1,6-P_2ase. [c] Numbers in parentheses indicated the n_H value.

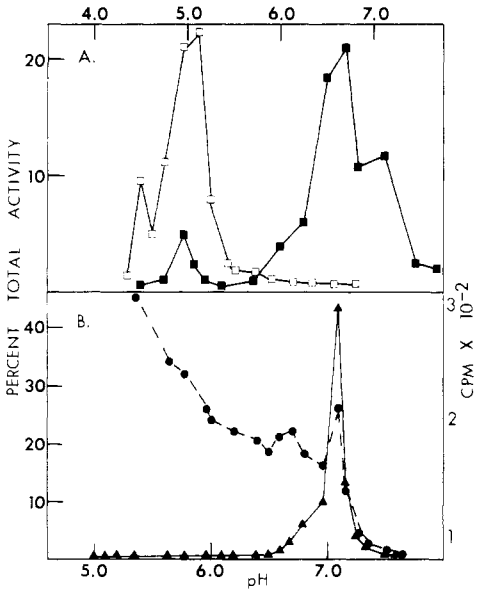

FIGURE 2. Typical pyruvate kinase activity profiles obtained from liver extracts. In panel A the ■ show data obtained from a fresh untreated extract. In about half the experiments, the ratio between pH 7.2 and 6.6 enzymes was reversed. The □ show data obtained with an extract dialyzed against excess EDTA. In panel B the ▲ illustrates pyruvate kinase activity values obtained from a fresh liver extract focused in the presence of [^{14}C]fru-1,6-P_2. The ● illustrate cpm × 10^{-2} minus background. Counting was performed for 10 min.

as development proceeds beyond the 11th day of development. These two activity peaks increase in parallel fashion, the higher pI form always predominating. The failure of activity between pH 7.2 and 8.1 and between pH 8.1 and 8.9 to fall to the baseline suggests that still smaller activity peaks exist but are masked. As was the case with the younger embryos, small activity peaks were obtained at lower pI values. Moreover, a 15 day embryonic extract yielded traces of activity at both pH 4.9 and 4.5, suggesting that indeed two distinct forms of the enzyme exist with low pI values.

Attempts were made to test possible interconversion of some of these peaks. Electrofocusing a previously isolated pH 6.6 enzyme in the presence of fru-1,6-P_2 converted the enzyme to a pH 7.2 form. Incubation of another isolated pH 7.2 enzyme preparation with fru-1,6-P_2-ase (Ibsen and Trippet, 1972) generated pH 8.8 enzyme as detected by reelectrofocusing. Reelectrofocusing of a preparation having an activity peak at pH 7.2 resulted in much loss of activity and isolation of pH 4.6 enzyme only.

P-enolpyruvate saturation kinetics were performed on pH 7.2 enzyme isolated from both young and old embryos and on pH 8.1 and 8.8 enzymes obtained from 19 and 21 day embryos. Some of these data are summarized in Table II.[3] Kinetic behavior of pH 7.2 enzyme from younger and older embryos is indistinguishable. In the absence of effector, the mean $K_{0.5S}$ value was 0.12 mM and the n_H value 1.4. Ala and Phe acted as typical negative K-type effectors raising the $K_{0.5S}$ value without lowering the V_m. Ala tended to raise the n_H value while Phe did not. Ser and fru-1,6-P_2 acted as K-type activators. Trp and Mg-ATP had their most marked effects on the V_m. The former lowered it by about 50%, and the latter by 30%.

The enzymes having pI values of 8.1 and 8.9 and which were isolated from fresh extracts of older embryos had kinetic properties which differed markedly from those of the pH 7.2 enzyme (Table II). For both of these enzymes, the $K_{0.5S}$ value in the presence or absence of effector, other than Phe, was similar to that obtained in the presence of fru-1,6-P_2 for the pH 7.2 enzyme (0.020–0.025 mM). Moreover, rate vs. P-enolpyruvate concentration plots were not sigmoidal. Phe was a weak competitive-type inhibitor, while Trp was an atypical noncompetitive inhibitor, lowering the V_m some 50% but yielding convex-up double reciprocal plots.

Kinetic parameters associated with P-enolpyruvate saturation were also determined for the high pI peaks obtained from the apparently aberrant 8 day embryonic extract and on the pH 8.8 enzyme generated from pH 7.2 enzyme (Table II). The $K_{0.5S}$ values for the pH 8.2, 8.6, and 8.8 enzymes are 0.27, 0.18 and 0.17 mM, respectively; the n_H values are 2.2, 1.9, and 1.2. These limited data are sufficient to show that these high pI forms are kinetically more similar to the pH 7.2 enzyme than to the high pI forms isolated from fresh extracts of the more highly developed embryos.

Liver. Extracts prepared from embryonic (19 days post fertilization), young chick (5 and 7 days after hatching), and adult cock livers yielded indistinguishable electrofocusing patterns, which in turn were similar to the patterns obtained using extracts of whole embryos of less than 15 days development. In half the preparations, pH 7.2 enzyme predominated; in half, pH 6.6 enzyme did (Figure 2A). Extracts electrofocused in the presence of [^{14}C]fru-1,6-P_2 yielded both enzymatic activity and radioactivity at pH 7.2

[3] A more detailed description of much of the kinetic data is presented elsewhere (Ibsen and Marles, 1976).

Table III: Characteristics of Pyruvate Kinases Obtained from Extracts of Adult Liver.

Characteristic	Variant Forms			
Mean pH	4.84[a]	5.04[b]	6.65	7.18
pI value ± SE (n)	±0.19 (7)	± 0.31 (5)	±0.07 (8)	±0.05 (8)
Relative quantity in fresh extracts	>6%	>6%	10–85%	10–85%
$K_{0.5S}$ value, mM P-enolpyruvate				
No effector	0.26 (1.7)[c]	0.38 (1.8)	0.36 (1.6)	0.14 (1.4)
5 mM Ala	0.13 (1.8)	0.53 (1.6)	0.65 (2.0)	0.38 (1.8)
5 mM Phe	0.50 (2.0)	0.47 (0.8)	0.52 (1.6)	0.85 (1.3)
5 mM Ser	0.070 (2.3)	0.032 (1.0)	0.060 (0.9)	0.060 (1.2)
5×10^{-5} M fru-1,6-P_2	0.40 (1.2)	0.039 (0.9)	0.025 (0.8)	0.022 (0.9)

[a] Kinetic data derived from a preparation treated with EDTA. [b] Kinetic data derived from a frozen preparation. Similar values were obtained from an EDTA preparation. [c] Values in parentheses after the $K_{0.5S}$ values indicate the n_H values.

(Figure 2B). Lower pI peaks were also obtained in small yield from fresh extracts (Figure 2A). In one preparation, two such peaks were obtained, one at pH 5.0 and one at 5.4. Because of the wide variation in pI values obtained at about pH 5.0 and the simultaneous presence of two peaks in a single extract, it was considered probable that two activity bands existed in liver extracts. The mean pI values shown in Table III for these two bands were arrived at by assuming that peaks with pI values of 5.0 or less represented one type of enzyme, while those with pI values greater than 5.0 represented a second type. Too little activity was obtained from fresh extracts to permit further study of these forms. However, frozen extracts (−70 °C) tend to yield more, lower pI material. Aliquots of one extract were run before and after freezing. Before freezing 90% of the total activity was obtained at pH 7.2. Although freezing and thawing resulted in no loss of activity, the preparation focused after freezing yielded 50% of its activity at pH 5.4 and 40% at pH 6.6. This suggests that both the pH 6.6 and 5.4 peaks can arise from the pH 7.2 peak. Similarly, refocused pH 7.2 enzyme again yielded small quantities of pH 5.3 enzyme.

In a further attempt to generate low pI material, another liver preparation was divided into two aliquots. One was dialyzed for 18 h against a solution containing 50 mM triethanolamine buffer, pH 7.0, with 50 mM KCl and 5 mM $MgSO_4$. The other was dialyzed against a similar solution which also contained 10 mM EDTA. The preparation without EDTA yielded a typical liver pattern. The preparation dialyzed against EDTA yielded the majority of activity at pH 4.6 and 5.0 (Figure 2A).

P-enolpyruvate saturation kinetics were determined on the two lower pI forms and on pH 6.6 and 7.2 enzymes, using the same effectors as used with the embryonic extracts (Table III). As may be seen by comparing Tables II and III, the kinetic behavior of pH 7.2 enzyme from liver and embryos is very similar. The enzymes also behaved similarly with respect to Trp and Mg-ATP: 5 mM concentrations of these effectors lowered the V_m of the pH 7.2 liver enzyme 55 and 32%, respectively.

The pH 6.6 and 5.0 enzymes have similar kinetic properties (Table III), which differ from those of the pH 7.2 enzyme only in that the $K_{0.5S}$ and n_H values obtained in the absence of effector, or in the presence of Ala, are higher. Trp lowered the V_m of the pH 5.0 and 6.6 enzymes by 53 and 43%, respectively. The lowest pI form obtained from the EDTA treated extract (Figure 2A) also had distinct kinetic properties; i.e., it was neither activated by fru-1,6-P_2 nor inhibited by Ala (Table III). As discussed below, these properties are shared, in the qualitative sense, by the lowest pI form obtained from extracts of brain or chick thigh muscle (Tables IV and VI).

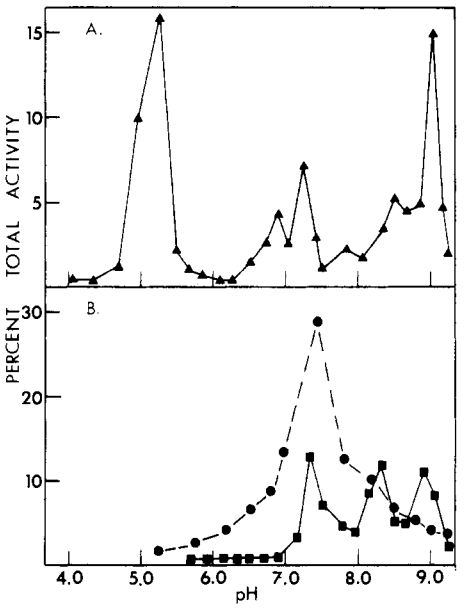

FIGURE 3. Profiles of pyruvate kinase activity obtained from extracts of adult brain (panel A) and heart (panel B). The ● illustrate data using a heart extract obtained from a 19 day embryo. The ■ illustrate data obtained from an extract prepared from an adult cock heart.

Brain. Two fresh and one frozen adult brain extract and one 7 day old chicken brain extract yielded activity profiles similar to the one illustrated in Figure 3A. At least five activity peaks always were obtained, at about pH 4.8, 7.3, 8.0, 8.5, and 9.0. In the case illustrated, a peak also was obtained at pH 6.9. The pH 4.8 peak was the major one but the pH 9.0 peak was also relatively large (Figure 3A). Table IV summarizes the mean pI values obtained for each peak and some salient kinetic parameters.

The pH 8.5 and 9.0 enzymes obtained from fresh brain extracts had relatively low $K_{0.5S}$ values and yielded hyperbolic rate plots. Phe and Ala acted as weak competitive inhibitors. The pH 4.8 peak also yielded essentially hyperbolic rate vs. P-enolpyruvate concentration plots, but had $K_{0.5S}$ values which are significantly higher than those obtained using pH 8.5 or 9.0 enzyme from fresh extracts (Table IV).

Table IV: Characteristics of Pyruvate Kinase Variants Obtained by Electrofocusing Brain Extracts.

Characteristic	Variant Forms						
Mean pH	4.82	~6.9	7.25	7.95	8.50	8.99	8.9[a]
pI value ± SE (n)	±0.18 (4)	(1)	±0.03 (4)	±0.12 (4)	±0.01 (4)	±0.06 (4)	(1)
$K_{0.5S}$ value, mM P-enolpyruvate							
No effector	0.08		0.056 (1.1)[b]		0.024	0.018	0.050 (0.9)
5 mM Ala	0.08		0.260 (1.4)		0.032	0.030	0.15 (1.3)
5 mM Phe	0.08 (<1.0)		0.450 (1.7)		0.024	0.028	0.16 (1.4)
5mM Ser	0.18		0.025		0.011	0.017	
5×10^{-5} M fru-1,6-P_2	0.10		0.020		0.016	0.020	0.11 (1.5)

[a] Generated from pH 4.84 enzyme by incubation for 30 min at 37 °C. [b] The number in parentheses after the $K_{0.5S}$ value is the n_H value. The absence of a parenthetical number indicates the $K_{0.5S}$ value was derived from a double reciprocal plot which was, at least as a first approximation, linear.

Table V: Characteristics of Pyruvate Kinase Variants Obtained from Heart Extracts.

Characteristic	Variant Forms		
Mean pH	7.49	8.1	8.9
pI value ± SE (n)	±0.08 (3)	(1)	(1)
When obtained	Embryo (19 day), chick, cock	Cock	Cock
$K_{0.5S}$ value, mM P-enolpyruvate			
No effector	0.045 (1.0)[a]	0.027 (1.0)	0.018 (1.0)
5 mM Ala	0.250 (1.3)	0.024 (1.0)	0.021 (1.0)
5 mM Phe	0.095 (0.9)	0.036 (1.0)	0.020 (1.0)

[a] The parenthetical numbers indicate the n_H value.

This low pI variant was relatively insensitive to Ala or Phe. One pH 4.8 peak was incubated at 37 °C for 30 min and then refocused; the majority of enzyme obtained by this treatment had a pI value of 8.9. Table IV summarizes kinetic constants obtained for the regenerated pH 8.9 peak. In the presence of Ala or Phe, sigmoidal rate vs. P-enolpyruvate concentration plots were obtained and, in general, the kinetic parameters were much different from those of the high pI peaks found in fresh extracts of late embryos or brain. Indeed, the kinetic properties are compatible with those of the pH 8.8 enzyme generated from embryonic pH 7.2 enzyme. Neither the pH 4.8 nor the pH 8.9 enzymes are activated by fru-1,6-P_2; in fact, the former enzyme may even be inhibited by both fru-1,6-P_2 and serine.

In the absence of P-enolpyruvate, the pH 7.3 activity peak yields essentially hyperbolic rate plots, but Ala and Phe act as typical K-type inhibitors increasing the $K_{0.5S}$ and n_H values to levels approaching those reported in Tables I and III for pH 7.2 enzyme. Fru-1,6-P_2 and Ser act as positive effectors.

When the pH 9.0 enzyme obtained from fresh extracts of brain was incubated and reelectrofocused, about 90% of the activity was recovered at pH 9.0.

Heart. Fresh heart extracts prepared from a 19 day old embryo, a 5 day old chick, and a cock were electrofocused. The embryonic and 5 day chick heart extracts yielded one peak at pH 7.4. However, the adult heart yielded three peaks of similar activity at pH 7.4, 8.1, and 8.9 (Figure 3B). As may be seen from Table V, the pH 7.4 peak yielded kinetic parameters very similar to the pH 7.3 peak obtained from brain, and the pH 8.1 and 8.9 peaks yielded values similar to those obtained from the analogous peaks previously described.

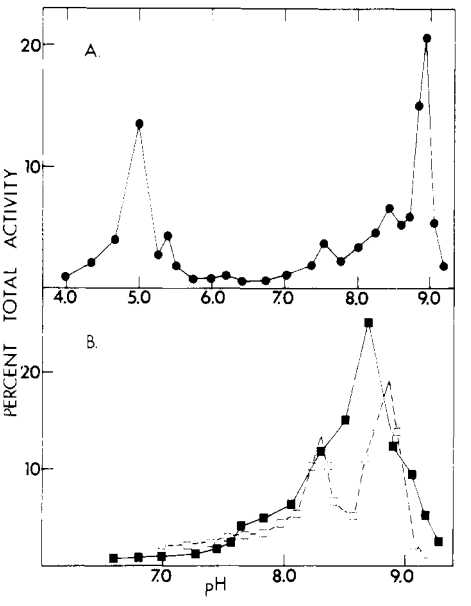

FIGURE 4. Pyruvate kinase activity profiles obtained from muscle extracts. Panel A illustrates results obtained from an extract prepared from all the thigh muscles of a 5 day old chick. In panel B the ■ represent data obtained from adult cock breast muscle, pectoralis major, and the □ represent data obtained from adult thigh muscle, mainly the semimembranosus.

Skeletal Muscle. Figures 4A and 4B and Table VI summarize data obtained using muscle extracts. An extract prepared from the thigh muscle of a 5 day old chicken yielded a complex pattern similar to that obtained from brain extracts. Kinetic analysis of the pH 4.9 and 8.5 peaks showed these forms to be similar to the analogous forms previously isolated from fresh extracts.

Adult thigh muscle extracts yielded only two activity peaks, at pH 8.0 and 8.8, suggesting that the lower pI forms are lost during development (Figure 4B). Adult breast muscle yields only one major band of activity at pH 8.8, but the asymmetric nature of the band suggests smaller peaks of activity are masked. Traces of activity were also found at pH 5.3 and 6.6. Analysis of the pH 8.0 and 8.8 peaks showed them to be of the low $K_{0.5S}$ type (Table VI).

Testes. Extracts prepared from testes yielded one symmetrical activity peak at pH 7.3. Kinetic analysis showed this peak to have kinetic properties similar to the pH 7.3–7.5 enzyme obtained from brain and heart. The mM $K_{0.5S}$ and n_H values for P-enolpyruvate are: 0.05 and 1.1, respectively in the absence of added effectors; 0.18 and 1.5 in the

Table VI: Characteristics of Pyruvate Kinase Variants Obtained from Muscle Extracts.

Characteristic	Variant Forms						
Mean pH	4.9	5.2	6.5	7.5	7.96	8.51	8.76
pI value ± SE (n)	(1)	(2)	(2)	(1)	±0.13 (3)	±0.04 (3)	±0.05 (5)
Relative amount obtained and source	Chick thigh (large)	Chick thigh (small), cock breast (trace)	Chick thigh (trace) cock breast (trace)	Chick thigh (small)	Cock thigh (large)	Chick thigh (small)	Chick thigh (large) cock thigh (large) cock breast (major)
$K_{0.5S}$ value, mM P-enolpyruvate							
No effector	0.060				0.022	0.037	0.022
5 mM Ala	0.050				0.025	0.054	0.050
5 mM Phe	0.048				0.033	0.080	0.071
5×10^{-5} M fru-1,6-P_2					0.033		0.019

[a] All values derived from basically linear double reciprocal plots.

presence of 5 mM Ala; 0.10 and 1.8 in the presence of 5 mM Phe; 0.024 and 1.2 in the presence of 5 mM Ser; and 0.027 and 0.9 in the presence of 5×10^{-5} M fru-1,6-P_2.

Discussion

Overview. Table VII summarizes the isoelectric variants obtained and some of their more obvious kinetic properties as well as their proposed quaternary structure. As was also true in the rat (Ibsen and Trippet, 1972), at least six variant forms of the K isozyme (which appear as a low pI set, an intermediate pI set and a high pI set) have been isolated. The intermediate pI set is the one most commonly derived from fresh extracts; the lower and higher pI sets, although sometimes present in fresh extracts, are generally found in increasing quantities after handling. Although the higher pI set of the K isozyme has pI values similar to the suspected M_4 and/or M_3K forms, they are readily distinguished kinetically. Therefore, both kinetic and electrofocusing data are required for identification. Similarly the proposed pH 7.2 K_4 and pH 7.4 K_3M forms can only be clearly distinguished by electrofocusing and kinetic data. Support for the identity of these various pI forms is supplied in the discussion below.

Properties and Distribution of the K Isozyme. Since the pH 7.2 (mean pI value 7.18) activity peak predominates in younger embryos (Figure 1), it might be related to the isozyme of the mammalian embryo. Therefore, it too will be called a K isozyme. Incubation of the less commonly isolated pH 6.6 enzyme (mean pI value 6.64) with fru-1,6-P_2 converted it to the pH 7.2 variant. This suggests that the pH 6.6 enzyme represents another form of the K isozyme, possibly the T conformer. Studies on rat (Ibsen and Trippet, 1972; Ibsen et al., 1975b) and mouse (Ibsen et al., 1975a) extracts show the form of the K isozyme predominating in fresh extracts is also derived in two interconvertible forms. As in the chick, fru-1,6-P_2 converts the lower pI form to the higher pI form, which also binds [^{14}C]fru-1,6-P_2. Both of these intermediate pI forms of the rat K isozyme also have the kinetic properties expected of an R,T set and molecular weights in the 200 000-dalton range (Ibsen and Trippet, 1972; Ibsen et al., 1975b).

Fresh extracts of fetal or adult chicken liver show predominant activity peaks at pH 6.6 (mean 6.65) and/or pH 7.2 (mean 7.18) (Figure 2). The similarity of these pI values to those obtained from embryonic extracts suggest that the major adult liver isozyme is also K type; i.e, that chicken liver does not differentiate to form an L isozyme as does mammalian liver. Further evidence of identity is shown by the fact that the two pH 7.2 enzymes have indistinguishable kinetic characteristics (Tables II and III). The conclusion that adult chicken liver retains the embryonic K isozyme is consistent with the observations of Cardenas et al. (1975b) and Strandholm et al. (1975).

The pH 6.6 enzyme tends to have the higher $K_{0.5S}$ values for P-enolpyruvate which is consistent with its being the T conformeric form (i.e., presumably, the pH 7.2 enzyme was isolated in the R form and was largely, but not fully, converted to the T form during the 5-min preincubation associated with the assay). Electrofocusing in the presence of ^{14}C-fru-1,6-P_2 clearly demonstrates an association between fru-1,6-P_2 and pH 7.2 enzyme (Figure 2B), again indicating it is the R conformer. A smaller peak of radioactivity was also obtained at about pH 6.6. As would be expected from a preparation run in the presence of fru-1,6-P_2, little pyruvate kinase activity was obtained at this pH value. These data might indicate, however, that the pH 6.6 enzyme also binds fru-1,6-P_2. If so, the ratio of radioactivity to enzyme activity is much greater than for the apparently more stable pH 7.2 complex. On the other hand, the pH value of this peak of fru-1,6-P_2 corresponds to an anomalous peak obtained from rat liver and kidney cortex extracts (Ibsen et al., 1975b).

Both pH 6.6 and 7.2 enzymes are homotropically activated by P-enolpyruvate and heterotropically by fru-1,6-P_2 and Ser. These K-isozyme variants also are strongly inhibited by Phe, Trp, and Ala. These characteristics are shared by the analogous rat K-isozyme variants but not by rat L or M isozymes (Ibsen and Trippet, 1974). Thus again, the rat and chick K isozymes behave similarly. Moreover, the conclusion that the enzyme obtained in extracts of chicken liver is not L type is further supported.

As was also demonstrated for the corresponding rat K-isozyme variants (Ibsen and Trippet, 1972), the chick pH 6.6 or 7.2 enzyme could be converted to at least two lower and two higher pI forms (Tables II and III). A slight difference between the chick and mammalian systems was that, whereas higher pI forms routinely were found in fresh rat or mouse extracts (Ibsen and Trippet, 1972; Ibsen et al., 1975a,b; Criss, 1969; Yanagi et al., 1971; Nakamura et al., 1972, 1974), they were observed in only one fresh chick extract from an 8 day embryo. However, a high pI form of the K isozyme (presumably equivalent to the highest pI form obtained from the apparently aberrant 8 day embryo) could

Table VII: Summary of the Pyruvate Kinase Variants Isolated and Their Proposed Relationships.

	K Isozyme Variants									
	Low pI Forms		Intermediate pI Forms		High pI Forms		M Isozyme	Hybrid Forms		
pI value	4.8	5.1	6.6	7.2	8.2		8.8	7.4	7.9	8.5
Range	4.4–4.9	4.9–5.4	6.5–6.7	7.1–7.3		8.6–8.9	8.7–9.0	7.2–7.5	7.8–8	8.5
Suspected subunit structure	K	K_2	K_4 (T form)	K_4 (R form)	K	K	M_4 8.0	K_3M	K_2M_2	KM_3
Gross kinetic properties										
No effector	S or h[a]	S	S	S	S	S or h	H	h	H	H
Alanine	0[b]	–	–	–		–	0	–	0	0
fru-1,6-P_2	0 or –	+	+	+		–	0	+	0	0
Serine	0 or +	+	+	+			0	+	0	0

[a] S indicates sigmoidal kinetics; h indicates not obviously sigmoidal with a relatively high $K_{0.5S}$ value (0.05 mM or greater); H indicates hyberbolic with a $K_{0.5S}$ of less than 0.04 mM. [b] 0 indicates no gross effect; – indicates a negative effect which increases the $K_{0.5S}$ and n_H value; + indicates a positive effect which decreases the $K_{0.5S}$ and n_H values. A blank space indicates no data are available.

be generated from both pH 7.2 enzyme (Table II) and pH 4.8 enzyme (Table IV).

The similarities in developmental patterns and in kinetic and physical properties suggest the chicken and mammalian K isozymes are homologous.

Relationship among K-Isozyme Variants. High pI isozyme variants appear to be the most common form obtained after electrophoresis in Mg^{2+}-containing buffers (Ibsen and Krueger, 1973). Moreover, the rat K isozyme elutes from DEAE-cellulose as if it were a high pI form (i.e., as if it were cationic at pH 7.0–7.5). Indeed, passage of an intermediate pI form of the rat K isozyme through DEAE appears to convert it to a high pI variant (Ibsen and Trippet, 1972). This observation may explain the somewhat distinctive kinetic properties of enzyme separated using DEAE (e.g., Costa et al., 1972). Reelectrofocusing studies show that both rat and chick high pI variants can be formed from either low or intermediate pI forms (Tables II and IV; Ibsen and Trippet, 1972). Although the mechanism of conversion is not clear, conceivably an oxidation is involved (see e.g., Badwey and Westhead, 1975).

That EDTA treatment induces formation of a low pI variant (Figure 2) suggests that their presence is associated with loss of bound Mg^{2+}. This mechanism is also consonant with the observations that more, low pI enzyme is formed after freezing and thawing or by reelectrofocusing. Low pI forms of the rat K isozyme have molecular weights of ~100 000 daltons (Ibsen and Trippet, 1972; Ibsen et al., 1975b).

Pogson (1968a,b) and Van Berkel (1974) have isolated a form of the rat K isozyme which is produced by Mg^{2+} deprivation. This variant, called the A form, travels as an anion (suggesting it has a low pI value) and sediments as if it had a molecular weight of ~100 000 (Pogson, 1968a). The kinetic properties (Pogson 1968b; Van Berkel, 1974) are qualitatively similar to those of the pH 5.0 chicken variant. These data suggest that the A variant described by these workers and the next to lowest pI form isolated by electrofocusing are identical. It is further hypothesized that incubation in the presence of Mg^{2+} converts this dimeric form A of the enzyme to the T conformer of the tetrameric form, i.e., to the lower pI form of the intermediate pI set (Table VII). This conversion is suggested by the following observations: when the lower pI form of the enzyme is subjected to kinetic studies initiated by addition of enzyme, higher and more variant $K_{0.5S}$ values for P-enolpyruvate are obtained, suggesting the dimeric form has intrinsic activity (Ibsen and Trippet, 1972); when the same enzyme is preincubated with Mg^{2+} containing solutions as under assay conditions, the kinetic properties are nearly identical with those obtained starting with the tetrameric form (e.g., Table III), suggesting tetramer is being assayed; and Mg^{2+} is able to convert apparent dimers to tetramers (Ibsen et al., 1975).

Van Berkel (1974) further reports that the A variant spontaneously converts to a third form. Conceivably, this would represent the lowest pI form. It may be that there are, in fact, a family of low pI forms. This is suggested by the somewhat disparate kinetic properties and the range of pI values (Table VII). Similar variability with the respect to lower pI forms was observed in extracts of rat tissue (Ibsen and Trippet, 1972).

Walker and Potter (1973) also identify two forms of the K isozyme in extracts from cultured liver cells. The so-called B form obtained from cells grown in the presence of glucose had a low $K_{0.5S}$ value and yielded hyperbolic rate plots, while the A form was obtained from cells grown in the absence of glucose and yielded sigmoidal rate plots. Because of the kinetic similarity, Walker and Potter proposed that these enzymes were equivalent to the B and A forms of Pogson. While the relationship between B forms seems probable, it seems possible that Walker and Potter's A form is equivalent to the proposed tetrameric T form. This supposition is supported by the following: fru-1,6-P_2 drives the T form to the R form, apparently without need of a dimeric intermediate (Figure 1; Ibsen et al., 1975b); addition of glucose to whole cells rapidly raises the fru-1,6-P_2 concentration to a level more than sufficient to activate the enzyme (e.g., Ibsen and Schiller, 1971); in the rat, starvation promotes a transition from the R to T forms, apparently without forming dimers (Ibsen and Trippet, 1972); and Walker and Potter (1973) noted fru-1,6-P_2 could convert their A form to the B form without adding Mg^{2+}, although Mg^{2+} did act synergistically.

The tendency of the middle pH (tetrameric) forms to give rise to lower and higher pI (probably dimeric) forms (Ibsen and Trippet, 1972; Ibsen et al., 1975b) suggests an asymmetric cleavage of the enzyme. Moreover, in the rat the low pI forms have pI values similar to those of the L isozyme, and the high pI variants have pI values similar to those of the M isozyme, suggesting that the rat tetramer is a heterologous dimer of dimers, as had been suggested (Tanaka et al., 1967; Bigley et al., 1968). However, this conclusion was not consistent with the pattern of interconvertibility (Ibsen and Trippet, 1972). Further evidence that high

and low pI forms are not L and M subunits isolated from an L_2M_2 hybrid is obtained by the studies reported herein, since the chicken seemingly lacks L isozyme, but the K isozyme still yields high and low pI forms. Nonetheless, the data still suggest an asymmetric cleavage, the most documented aspect of which is the tendency of the T conformer to yield lower pI, dimeric forms. This cleavage is accentuated by chelators and the process is reversed by Mg^{2+}. These data, plus the observations which suggest it is the higher pI tetrameric conformer which has bound fru-1,6-P_2, suggest that the T-conformational tetramer is stabilized by Mg^{2+} in a way which fully neutralizes the charge. In contrast, in the R conformation, the Mg^{2+} charge is not fully neutralized. This could account for the unexpected tendency of fru-1,6-P_2 to raise the pI value as well as the greater susceptibility of the T conformer to cleavage.

Although the pleomorphism of the pyruvate kinase isozymes can no longer be disputed, it is not clear how the enzyme exists in vivo. Implicit in this discussion and in previous reports concerning the mammalian enzymes is the assumption that the predominant forms obtained by electrofocusing fresh extracts represent the true in vivo forms, whereas the forms which increase in quantity after manipulation are derived forms. Yet, other workers have obtained greater proportions of higher pI enzyme after electrofocusing fresh extracts. Moreover, evidence has been obtained for the presence of multiple forms (other than R,T sets) in fresh extracts (Ibsen and Trippet, 1972; Boivin et al., 1972; Van Berkel et al., 1974) or for different variants in different tissues (Tanaka et al., 1970). The human red cell enzyme also appears to change its physical properties when aged under blood bank conditions (Ibsen et al., 1971) or in the body (Paglia et al., 1970). Possibly the enzyme is programmed for destruction if held in vivo in the T conformation for extended periods. Certainly the T conformer is less stable in vitro (Ibsen and Trippet, 1973).

Tissues having multiple forms of non-K-type isozyme (notably brain and young chick thigh muscle) yield primarily the lowest pI form of the K isozyme upon electrofocusing (Figures 3A and 4). The significance of this observation is not clear, but a similar pattern was observed in Xenopus tissue extracts (Ibsen et al., to be published).

M Isozyme. Cardenas et al. (1975a) and Strandholm et al. (1975) obtained a band of activity from chicken brain and muscle which migrated more slowly than did the K isozyme and obtained evidence indicating this enzyme was the homologue of mammalian M isozyme. Cardenas et al. (1975a) found the purified chicken breast muscle enzyme to have a pI value of 8.77 and adduced kinetic and physical evidence indicating this enzyme to be a homotetrameric (M_4) enzyme. The pI values of the highest pI form (pH 8.8–9.0) found in fresh extracts of adult brain, heart, and muscle and in the more developed embryos are compatible with these forms being the M_4 isozyme studied by Cardenas et al. (1975a). Moreover, the adult tissues showing this high pI form are the same tissues which have the M isozyme in mammals.

In addition, the mammalian M_4 isozymes, the purified pH 8.77 chicken enzyme, and the 8.8–9.0 enzymes isolated in these studies have similar kinetic properties. In the absence of effector, hyperbolic rates plots are obtained and the $K_{0.5S}$ values are low. In a study of highly purified chicken M_4 enzyme, Cardenas et al. (1975a) obtained a $K_{0.5S}$ value of 0.04 mM for P-enolpyruvate, while in this study the pH 8.8–9.0 enzyme from electrofocused extracts had $K_{0.5S}$ values of about 0.02 mM. This difference could well be related to the different pH and ADP levels used in the two studies. That Leveille (1969) using crude extracts, obtained still higher $K_{0.5S}$ values suggests the difference is not related to the amount of purification.

Table VIII: Summary of Recombination Experiments.

Preparations Used[a]	Recovered Activity pH	Recovered Activity % of Total	$K_{0.5S}$ mM P-enol-pyruvate	Suspected Isozyme
Liver pH 7.2	7.4	11.3	0.05	K_3M
peak plus	8.0	37.5	0.10	K_4
breast muscle	8.5	50.4	0.03	KM_3
pH 8.8 peak				
(K_4 plus M_4)				
Embryo pH 7.2	5.5	2.6		K_4
peak plus	7.1	6.9	0.14	K_4
chick thigh muscle	8.0	17.3	0.06	K_2M_2
pH 8.9 peak	8.6	32.2	0.03	KM_3
(K_4 plus M_4)	9.0	35.7	0.03	M_4
7 day chick liver[b]	6.5	25.5		K_4
pH 7.2 peak	7.2	11.5		K_4 or K_3M
plus	8.0	11.5		K_2M_2
brain pH 8.9 peak	8.4	10.2		KM_3
(K_4 plus M_4)	8.8	18.9		M_4
Adult liver[b]	6.6	35.5		K_4
pH 7.2 enzyme	7.2	22.1		K_4
plus	7.6	10.6		K_3M
brain pH 8.9	8.8	10.6		M_4
(K_4 plus M_4)				
Thigh muscle	7.4	22.7	0.066	K_3M
pH 8.0 peak	7.8	34.3	0.040	K_2M_2
(K_2M_2)	8.3	20.0	0.026	KM_3
	8.7	32.3	0.026	M_4

[a] All preparations were mixed in the presence of guanidine–HCl until activity was lost. They were then dialyzed as described in the methods section. [b] Total activity recovered was only about 10%.

Ser, Ala, and Phe have but a minor effect on the chicken M isozyme (Tables IV–VI). However, the Phe- and Trp-inhibited enzyme is activated by Ala (Ibsen and Marles, accompanying manuscript). This activating effect of Ala also has been noted for the rat M isozyme (Carminatti et al., 1971; Ibsen and Trippet, 1974).

The low $K_{0.5S}$ values, the absence of homotropic activation effects, and the relative lack of susceptibility of Ala or Ser inhibition provide kinetic characteristics which clearly distinguish the suspected M_4 isozyme from the high pI variants of the K isozyme. Moreover, it was not possible to generate K-variant forms from low $K_{0.5S}$, high pI enzyme.

In summary, these data indicate that the pH 8.8–9.0 enzyme: represents an isozyme distinct from any of the K forms; has kinetic properties and tissue distribution patterns similar to those of the mammalian M isozyme; and has kinetic properties and pI value similar to the M_4 isozyme studied by Cardenas et al. (1975a). These observations support the concept that this low $K_{0.5S}$, pH 8.8–9.0 enzyme from chicken tissues is the homologue of the mammalian M_4 isozyme.

KM Isozymes. In an electrophoretic survey of the pyruvate kinases of chicken tissues, Cardenas et al. (1975b) and Strandholm et al. (1975) found only two different activity bands. In contrast, electrofocusing yields at least ten activity bands, six of which are K-isozyme variants and one the M isozyme. The remaining bands of activity are: a low $K_{0.5S}$, pH 8.5 enzyme found in muscle and brain extracts; a

low $K_{0.5S}$ pH 7.8–8.1 band found in the more developed embryonic extracts and in brain, muscle, and heart extracts; and an intermediate $K_{0.5S}$, pH 7.3–7.5 enzyme obtained in extracts of muscle, brain, heart, and testes. Although the pI value of this latter enzyme is within experimental error of the range of values obtained from the pH 7.2 K isozyme, the $K_{0.5S}$ values obtained in the absence of effectors appears to be significantly different. Moreover, hyperbolic rate plots are obtained. Thus there appear to be five pI forms commonly isolated from fresh tissue extracts of organs having the M_4 enzyme, namely: the pH 6.6, 7.2 forms, believed to be K_4; a pH 7.3 to 7.5 (7.4) form; a pH 7.8 to 8.1 (7.9) form; a pH 8.5 form; and a pH 8.8 to 9.0 (8.9) form, believed to be an M_4 isozyme. The roughly 0.5 pH unit increments of pI values suggest the presence of hybrid forms; i.e, pH 7.4 enzyme would be K_3M, pH 7.9 enzyme K_2M_2, and pH 8.5 enzyme KM_3. The kinetic properties of these forms are compatible with this concept inasmuch as the pH 6.6–7.2 set tends to have the highest $K_{0.5S}$ and n_H values, and the pH 8.8–9.0 forms the lowest. However, this progression is not proportional. There is a more marked change in kinetic properties between the pH 7.4 and the 7.9 forms than between any of the others. That is, kinetically the suspected K_2M_2 enzyme resembles the M_4 enzyme much more than it does the K_4 enzyme. Dyson and Cardenas (1973) and Cardenas et al. (1975b) found a somewhat analogous relationship among the purified bovine L_4 and M_4 enzymes and their hybrids formed in vitro.

Table VIII summarizes the results of a series of experiments designed to determine if similar hybrid forms can be formed in vitro. The data are somewhat variable, possibly because the experiments were performed with partially purified preparations, but dissociation and reassociation of mixtures of K_4 and M_4 isozymes or of the K_2M_2 isozyme generate new forms with the kinetic properties and pI values of the suspected hybrid forms. These data reinforce the concept that the intermediate pI forms are hybrids. When the dissociation agent, guanidine–HCl, was not added, but the enzyme fractions were otherwise treated identically, only one bit of evidence for the formation of a hybrid was obtained, namely: in one of the experiments run without guanidine–HCl in parallel with those reported in Table VIII, a pH 8.2 variant having a low $K_{0.5S}$ value for P-enolpyruvate was isolated. On the other hand, no evidence for hybrid forms was obtained when liver or breast muscle tissues were mixed prior to homogenization and then treated as usual. This suggests that the hybrids were not formed during isolation and that, if the intermediate pI forms are hybrids, they are formed in vivo, within the same cell (Garnett et al., 1974).

That no evidence for intermediate pI forms was obtained in the electrophoretic studies of Cardenas et al. (1975b) and Strandholm et al. (1975) may be due to limitations in the electrophoretic methods. In the first place the K_4 and M_4 forms migrate on cellulose acetate at similar rates, and a minimal separation was obtained even after 15 h of electrophoresis. Secondly, the method of visualization employed (i.e., disappearance of NADH fluorescence) has an inherent bias against minor bands in juxtaposition to major ones. An alternate, but unlikely, possibility is that there is difference between the Oregon and California White Leghorns. Such a possibility is made even more remote by the fact that intermediate pI bands also were obtained from Rhode Island Red–White Leghorn hybrids. Still additional, but remote, possibilities for this apparent discrepancy are that the M_4 enzyme is also highly pleomorphic or that the K isozyme can exist in still more forms. Some evidence of pleomorphism for the rat M isozyme was obtained (Ibsen and Trippet, 1972), but the changes in pI value observed were less than 0.2 of a pH unit. Conceivably the same sort of pleomorphism in the M isozyme may have been observed in these studies; e.g., brain M_4 isozyme tends to have a slightly higher pI value than muscle M_4 isozyme (Tables IV and VI). It is interesting to note that Cardenas et al. (1975a) obtained a relatively small activity peak at pH 8.45 when their purified, apparently homotetrameric, M_4 isozyme was electrofocused. They ascribed this to microheterogeneity. Conversely, it may be that some M_3K isozyme was copurified with the M_4 isozyme.

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References

Badwey, J. A., and Westhead, E. W. (1975), Isozymes I. Molecular Structure, Markert, C. L., Ed., New York, N.Y., Academic Press, p 509.

Bailey, E., and Walker, P. R. (1969), *Biochem. J. 111.* 359.

Balinsky, D., Cayanis, E., and Bersohn, I. (1973), *Biochemistry 12,* 863.

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

Boivin, P., Galand, C., and Demartial, M. C. (1972), *Nouv. Rev. Fr. Hematol. 12,* 159.

Carbonell, J., Feliu, J. E., Marco, R., and Sols, A. (1973), *Eur. J. Biochem. 37,* 148.

Cardenas, J. M., Blachly, E. G. Ceccotti, P. L., and Dyson, R. D. (1975a), *Biochemistry 14,* 2247.

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. (1975b), Isozymes I. Molecular Structure, Markert, C. L., Ed., New York, N.Y., Academic Press, p 523.

Carminatti, H., Jiménez de Aśua, L., Liederman, B., and Rozengurt, E. (1971), *J. Biol. Chem. 246,* 7284.

Costa, L., Jiménez de Aśua, L., Rozengurt, E., Bade E. G., and Carminatti, H. (1972), *Biochim. Biophys. Acta 289,* 128.

Criss, W. E. (1969), *Biochem. Biophys. Res. Commun. 35,* 901.

Dyson, R. D., and Cardenas, J. M. (1973), *J. Biol. Chem. 248,* 8482.

Farina, F. A., Shatton, J. B., Morris, H. P., and Weinhouse, S. (1974), *Cancer Res. 34,* 1439.

Friedenthal, M., Roselino, E., and Passerson, S. (1973), *Eur. J. Biochem. 35,* 148.

Gabrielli, F., and Baldi, S. (1972), *Eur. J. Biochem. 31,* 209.

Garnett, M. E., Dyson, R. D., and Dost, F. N. (1974), *J. Biol. Chem. 249,* 5222.

Ibsen, K. H., Basabe, J. R., and Lopez, T. P. (1975a), *Cancer Res. 35,* 180.

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

Ibsen, K. H., and Marles, S. W. (1976), *Biochemistry* (the following paper in this issue).

Ibsen, K. H., and Schiller, K. W. (1971), *Arch. Biochem. Biophys. 143,* 187.
Ibsen, K. H., Schiller, K. W., and Haas, T. A. (1971), *J. Biol. Chem. 246,* 1233.
Ibsen, K. H., and Trippet, P. (1972), *Biochemistry 11,* 4442.
Ibsen, K. H., and Trippet, P. (1973), *Arch. Biochem. Biophys. 156,* 730.
Ibsen, K. H., and Trippet, P. (1974), *Arch. Biochem. Biophys. 163,* 570.
Ibsen, K. H., Trippet, P., and Basabe, J. (1975b), Isozymes I. Molecular Structure, C. L. Markert, Ed., New York, N.Y., Academic Press, p 543.
Imamura, K., and Tanaka, T. (1972), *J. Biochem. (Tokyo) 71,* 1043.
Imamura, K., Tanaka, T., Nishina, T., Nakashima, K., and Miwa, S. (1973), *J. Biochem. (Tokyo) 74,* 1165.
Kayne, F. J. (1973), *Enzymes, 3rd Ed. 8,* 353.
Kornecki-Gerrity, E. H., and Penny, D. G. (1974), *Comp. Biochem. Physiol. B: 49,* 15.
Kozhevnikovn, K. A. (1973), *Biokhimiya 38,* 670.
Krebs, H. A., and Eggleston, L. V. (1965), *Biochem. J. 94,* 3C.
Leveille, G. A. (1969), *Comp. Biochem. Physiol. 28,* 733.
Malcovati, M., Valentini, G., and Kornberg, H. L. (1973), *Acta Vitaminol. Enzymol. 27,* 96.
Mustafa, T., and Hochachka, P. W. (1971), *J. Biol. Chem. 246,* 3196.
Nakamura, T., Fujii, M., and Kumegawa, M. (1974), *Arch. Oral. Biol. 19,* 645.
Nakamura, T., Hosoi, K., Nishikawa, K., and Horio, T. (1972), *Gann 63,* 239.
Osterman, J., and Fritz, P. J. (1973), *Comp. Biochem. Physiol. B: 44,* 1077.
Osterman, J., Fritz, P. J., and Wuntch, T. (1973), *J. Biol. Chem. 248,* 1011.
Paglia, D. E., Valentine, W. N., Kurschner, K. K., and Wilson, M. L. (1970), *J. Lab. Clin. Med. 76,* 202.
Peterson, J. S., Chern, C. J., Harkins, R. N., and Black, J. A. (1974), *FEBS Lett. 49,* 73.
Pogson, C. I. (1968a), *Biochem. Biophys. Res. Commun. 30,* 297.
Pogson, C. I. (1968b), *Biochem. J. 110,* 67.
Schloen, L. H., Kmiotek, E. H., and Sallach. H. J. (1974), *Arch. Biochem. Biophys. 164,* 254.
Schulz, J., and Sparmann, G. (1972), *Wiss. Z., Karl-Marx-Univ., Leipzig, Math.-Naturwiss. Reihe 21,* 631.
Somero, G. N., and Hochachka, P. W. (1968), *Biochem. J. 110,* 395.
Strandholm, J. J., Cardenas, J. M., and Dyson, R. D. (1975), *Biochemistry 14,* 2242.
Suda, M., Tanaka, T., Yanagi, S., Hayashi, S., Imamura, K., and Tanuichi, K. (1972), *GANN Monogr. Cancer Res., No. 13,* 79.
Susor, W. A., and Rutter, W. J. (1968), *Biochem. Biophys. Res. Commun. 30,* 14.
Susor, W. A., and Rutter, W. J. (1971), *Anal. Biochem. 43,* 147.
Tanaka, T., Harano, Y., Sue, F., and Morimura, J. (1967), *J. Biochem. (Tokyo) 62,* 71.
Tanaka, T., Imamura, K., Ann, T., and Taniuchi, K. (1970), Abstracts of the Proceedings of the 8th International Congress of Biochemistry, J. G. Gregory, Ed., Switzerland, p 227.
Tanaka, T., Yanagi, S., Miyahara, M., Kaku, R., Imamura, K., Tanuichi, K., and Suda, M. (1972), *Gann 63,* 555.
Van Berkel, T. J. C. (1974), *Biochim. Biophys. Acta 370,* 140.
Van Berkel, T. J. C., de Jonge, J. R., Koster, J. F., and Hulsmann, W. C. (1974), *Biochem. Biophys. Res. Commun. 60,* 398.
Walker, P. R., and Potter, V. R. (1973), *J. Biol. Chem. 248,* 4610.
Whittell, N. M., Ng, D. O. K., Prabhakararao, K., and Holmes, R. S. (1973), *Comp. Biochem. Physiol. B: 46,* 71.
Yamada, M. (1974), *Poult. Sci. 53,* 898.
Yanagi, S., Kamiya, T., Ikehara, Y., and Endo, H. (1971), *Gann 62,* 283.
Yanagi, S., Makiura, S., Arai, M., Matsumura, K., Hirao, K., Ito, N., and Tanaka, T. (1974), *Cancer Res. 34,* 2283.

Inhibition of Chicken Pyruvate Kinases by Amino Acids†

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ABSTRACT: Alanine, serine, and phenylalanine behave as inhibitors competitive with phosphoenolpyruvate for the activated forms of the chicken pyruvate kinases. On the other hand, phenylalanine and alanine behave as K-type inhibitors and serine behaves as a heterotropic activator of pyruvate kinase variants which undergo homotropic activation. Tryptophan lowers the V_m and tends to yield complex plots with all variants studied. Kinetic patterns obtained in the presence of phenylalanine also show some characteristics not generally associated with a competitive mechanism. These observations are related to data previously obtained using the rat isozymes and are used to formulate a mechanism which explains the effects of the amino acids. This mechanism hypothesizes that all the effector amino acids bind to the phosphoenolpyruvate site; however, amino acids with nonpolar side chains also interact with a nonpolar region of the T conformer and thereby stabilize it. It is further proposed that there are two such nonpolar regions on the various pyruvate kinases—the one which reacts with the nonbulky side chains, and another which reacts only with relatively bulky side chains. The stabilizing effect of this second nonpolar interaction imparts inhibitory characteristics which are not competitive in nature. Serine and perhaps other polar compounds may also bind at the phosphoenolpyruvate site, but because of their polarity exert a repulsive force at the same nonpolar site with which the nonbulky nonpolar amino acids interact. This repulsion stabilizes the R conformation. Presumably the homotropic activating ef-