Interconvertible and Noninterconvertible Forms of Rat Pyruvate Kinase†

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ABSTRACT: Kidney cortex pyruvate kinase was compared to heart and liver isoenzymes with respect to solubility in $(NH_4)_2SO_4$, isoelectric points, sedimentation, and kinetic properties. These data suggest that two-thirds of the activity in kidney cortex extracts is a pyruvate kinase molecularly distinct from muscle or liver isoenzymes. The probable *in vivo* form of this enzyme appears to exist as an R,T conformational pair with mean p*I* values of 6.77 and 6.41 and molecular weights of about 200,000. These conformers, whether in homogenates or partially purified, may be converted to forms of the enzyme with high $K_{0.5S}$ values for phosphoenolpyruvate, molecular weights of about 100,000 and mean p*I* values of 5.13, 7.50, or 7.70. The lower and higher molecular weight conformers were shown to be largely interconvertible. The remaining third of the activity in kidney cortex extracts had properties similar to a conformational variant of the major isoenzyme of liver. The major liver isoenzymes had molecular weights of about 200,000 and mean p*I* values of 5.46 and 5.73. A minor isoenzyme of liver had electrofocusing and kinetic properties similar to the distinct kidney enzyme. Isoelectric focused heart muscle enzyme yielded peaks of activity between pH 7.2 and 7.6. The conversion of the major kidney cortex enzyme from high molecular weight forms into forms with about half the weight of native enzyme and with p*I* values similar to liver or muscle enzyme suggested the native kidney enzyme existed as a hybrid containing muscle and liver enzymes. However, the high degree of interconvertibility exhibited by purified kidney variants ruled out this simple conclusion. This incongruity coupled to the observation that partially purified liver enzyme could be converted to a form similar to muscle enzyme suggest that the basic catalytic unit of the three mammalian isoenzymes have a common primary structure.

The pyruvate kinase (EC 2.7.1.40) in extracts of rat or human skeletal muscle, heart, brain, and leucocytes all have similar electrophoretic, immunological, and kinetic properties. Liver also may have this type "M" isoenzyme, but primarily contains an isoenzyme which is distinct by these same criteria (Tanaka *et al.*, 1965, 1967; Bigley *et al.*, 1968; Pogson, 1968; Susor and Rutter, 1968). This latter, type "L" isoenzyme further differs from the type M enzyme in that it appears to be induced by carbohydrate feeding or insulin administration (Tanaka *et al.*, 1965, 1967; Weber *et al.*, 1965a,b; Krebs and Eggleston, 1965). The genetic and kinetic properties of the L isoenzyme would appear to permit gluconeogenesis to occur while minimizing a futile energy consuming cycle between P-enolpyruvate[1] and pyruvate.

The kidney cortex is the second gluconeogenic organ of mammals. Thus one would expect extracts of kidney cortex to contain an L-type isoenzyme. The data available are contradictory. Rat kidney pyruvate kinase is completely inactivated by antiserum to type M isoenzyme (Tanaka *et al.*, 1967; Susor and Rutter, 1968), but upon electrophoresis it behaves as if it were either a mixture of L and M isoenzymes (Tanaka *et al.*, 1965, 1967; Pogson, 1968) or L isoenzyme and a third isoenzyme (Susor and Rutter, 1968, 1971). Kinetic data were interpreted to show that whole kidney extracts contain both L and M isoenzymes, while kidney cortex extracts contain only type L isoenzyme (Llorente *et al.*, 1970). DEAE chromatography of kidney cortex extracts yielded a band of activity with unique kinetic properties and a band which behaved like type L isoenzyme (Jiménez de Asúa *et al.*, 1971a). Human kidney enzyme had distinct properties which were consistent with the concept that it is a hybrid having both L and M subunits (Bigley *et al.*, 1968).

Electrofocusing, solubility, sedimentation, and kinetic data are presented which strongly support the concept that the pyruvate kinase of kidney cortex is a mixture of two enzyme forms, one of which is distinct from either type L or M isoenzyme and the other of which is an L-type isoenzyme. The data further show that each of the three apparently unique molecular forms may exist in several active conformations which are separable by electrofocusing. The kinetic and sedimentation properties of these conformers are described.

Experimental Procedure

Preparation of Tissue Extracts. Male Sprague–Dawley rats (approximately 200 g) were decapitated and exsanguinated. Organs were quickly iced, trimmed, and homogenized for 20–40 sec in iced 0.25 M sucrose with 7.2 mM 2-mercaptoethanol using a Thomas tissue grinder with a Teflon pestle at 200–500 rpm. The homogenates were centrifuged at 110,000*g* at 4° for 1 hr. The pH values of kidney cortex extracts were about 6.9; heart and liver extracts had pH values of about 6.8.

Assay and Kinetic Determination. The coupled lactate dehydrogenase system was used to determine pyruvate kinase activity as described (Ibsen *et al.*, 1971). In all kinetic determinations: the dehydrogenase was dialyzed against buffer prior to use; the ADP concentration was 0.2 mM; and the reaction was started by addition of less than 0.1 ml of cold enzyme to 1 or 3 ml of the reaction solution at 32°.

Sedimentation Studies. The sample and three internal standards (bovine serum albumin (mol wt 67,000), rabbit muscle lactate dehydrogenase (mol wt 136,500), and bovine

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[1] Abbreviations used are: fructose 1,6-diphosphate, Fru-1,6-di-P; fructose-1,6-diphosphate 1-phosphohydrolase, Fru-1,6-di-Pase; phosphoenolpyruvate, P-enolpyruvate.

TABLE I: Comparison of Kinetic and Sedimentation Properties of the Pyruvate Kinases Present in Extracts of Rat Kidney Cortex, Liver, and Heart.

Enzyme Source	Treatment	IU/mg of Protein	Kinetic Parameters: No Fru-1,6-di-P $K_{0.5S}$[a]	No Fru-1,6-di-P n_H	5 × 10⁻⁵ M Fru-1,6-di-P Activation[b]	5 × 10⁻⁵ M Fru-1,6-di-P $K_{0.5S}$[a]	5 × 10⁻⁵ M Fru-1,6-di-P n_H	App Mol Wt × 10⁻³ Mean	App Mol Wt × 10⁻³ ±SE	No. of Detm
Kidney Cortex	None[c]	0.44	1.8	1.5	15	0.70	1.0	178 116	9.6 8.3	4 4
	Incubated[d]	0.36	*f*	2.0	159	15.0 0.5	0.7	145	1.6	2
	Fasted[e]	0.43	6.2	1.8	30	0.6	1.0	179	5.4	4
Liver	None[c]	0.49	7.0	1.4	25	0.53	1.0	187	6.6	4
	Incubated[d]	0.41	4.0	1.5	45	0.52	1.0	216		1
	Fasted[e]	0.21	*f*	1.1	30	1.0 0.5	0.7	189	8.4	5
Heart	None[c]	1.56	0.55	1.0	1.5	0.55	1.0	191	9.9	3
	Incubated[d]	1.05	0.65	1.0	0.9	0.65	1.0	214		1
	Fasted[e]	0.86	0.50	1.3	1.2	0.59	1.3	209	0.4	2

[a] M × 10⁴ P-enolpyruvate. Conditions are described in the Methods section. [b] Activity with Fru-1,6-di-P divided by activity without Fru-1,6-di-P at 0.02 M P-enolpyruvate. [c] High-speed supernatant constantly maintained at 4° from *ad lib* fed animals. [d] As in *c* but supernatant incubated at 32° for 30 min at pH 7.0–7.2 in the presence of Fru-1,6-di-Pase. [e] As in *c* but from rats fasted 68–72 hr. [f] Apparent mixture of high and low $K_{0.5S}$ forms. Values reported for the activated form have been estimated as described (Ibsen *et al.*, 1971).

liver catalase (mol wt 247,000)) were layered on 4.6-ml linear 15 or 20 to 45% glycerol gradients and centrifuged at 40,000 rpm for 16.5 hr at 8° in a SW-50L rotor. For each tube the apparent molecular weights raised to the two-thirds power were plotted against fraction number (Martin and Ames, 1961). It was necessary to dilute samples to 20% or less glycerol. Standards and unknown samples yielded one peak unless otherwise noted.

Isoelectric Focusing. Electrofocusing experiments were usually conducted in an LKB 8101 column (110) with the anode at the bottom as previously described (Ibsen and Trippet, 1971). A few experiments were conducted with the anode at the top, but separation was poor, presumably due to the tendency to collect large quantities of precipitate at lower pH values.

The pH values were determined at 4° utilizing a Corning-Digital 111 pH meter coupled to a Thomas combination electrode No. 4858-L15. Extended exposure to Ampholine solutions tended to "fatigue" the electrode as indicated by failure to measure the correct pH of standard solutions, and an increased tendency to show a drift toward higher pH values. Such fatigued electrodes could be revitalized by soaking in 0.1 N HCl overnight, or if this failed by replacing the saturated KCl solution. Since some drift remained, all pH measurements were made after the probe had been immersed for 2 min. To further assure reliability of the pH readings all values represent the mean from two different Thomas electrodes, which agreed within 0.05 pH unit. In several of the latter experiments these values were also compared to those obtained utilizing a Beckman 39183 combination electrode.

Fru-1,6-di-Pase. About 0.1 unit (as defined by the vendor, P-L Biochemicals, Milwaukee, Wis.) was used in all incubations. Incubation of this quantity of enzyme at pH 7.0 and 32° with 5 × 10⁻⁵ M Fru-1,6-di-P in 3 ml destroyed the capacity of the Fru-1,6-di-P to activate pyruvate kinase within 5 min. The preparation had no discernible pyruvate kinase, adenylate kinase, or aldolase activity.

Protein. Protein was determined by the technique of Lowry *et al.* (1951).

Results

Properties of Pyruvate Kinase in Tissue Extracts. KIDNEY CORTEX. Some kinetic and physical properties of pyruvate kinase obtained from fresh and incubated high-speed supernatant solutions of tissue homogenates are summarized in Table I. Kidney cortex enzyme, continuously kept at 4°, was activated significantly by Fru-1,6-di-P. $K_{0.5S}$ values for P-enolpyruvate were 1.8 × 10⁻⁴ and 7.0 × 10⁻⁵ M in the absence and presence of Fru-1,6-di-P, respectively. Incubation at 32° for 30 min increased Fru-1,6-di-P activation. Even in the presence of Fru-1,6-di-P the incubated enzyme yielded kinetics suggesting the simultaneous presence of both a lower and a higher $K_{0.5S}$ form, having $K_{0.5S}$ values of about 5 × 10⁻⁵ and 10⁻³ M P-enolpyruvate, respectively.

As reported by Krebs and Eggleston (1965) fasting induced no reduction in specific activity. However, the data show that the $K_{0.5S}$ value for P-enolpyruvate was raised significantly when assayed in the absence of Fru-1,6-di-P (Table I). This change may represent a more complete conversion to an inactive (T) form of a conformational pair since assay in the presence of Fru-1,6-di-P completely converted it to the lower $K_{0.5S}$ form.

Sedimentation studies, also summarized in Table I, showed two peaks of activity for unincubated extracts with apparent molecular weights of 178,000 and 116,000, respectively. These two forms were found in varying proportions in different preparations but the more dense form predominated. Only

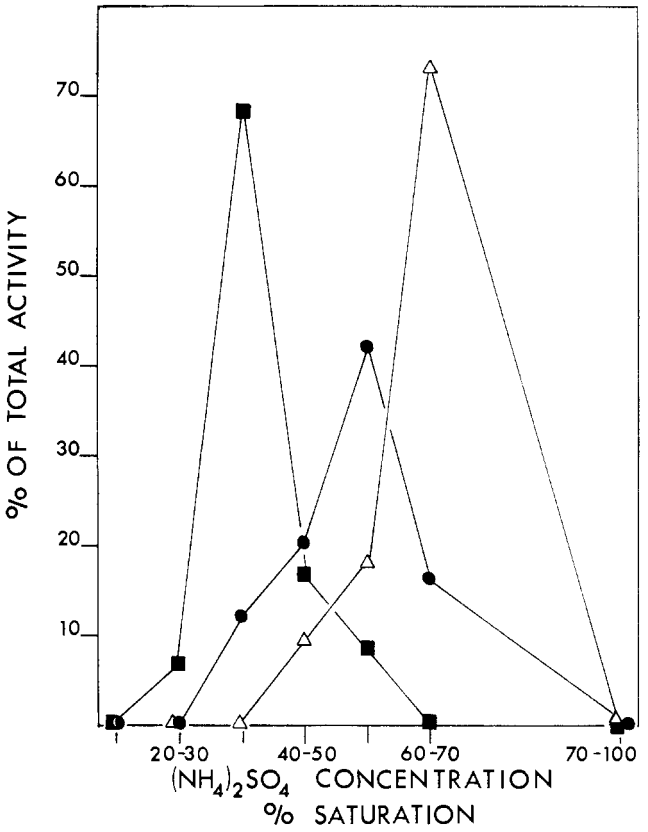

FIGURE 1: Relative solubility of rat kidney cortex, heart, and liver pyruvate kinases in solutions of increasing concentrations of $(NH_4)_2SO_4$. The data were obtained by subtracting the amount precipitated in each fraction from that of the preceding one. The data were obtained from liver extracts (■), from kidney cortex extracts (●), and from heart extracts (△).

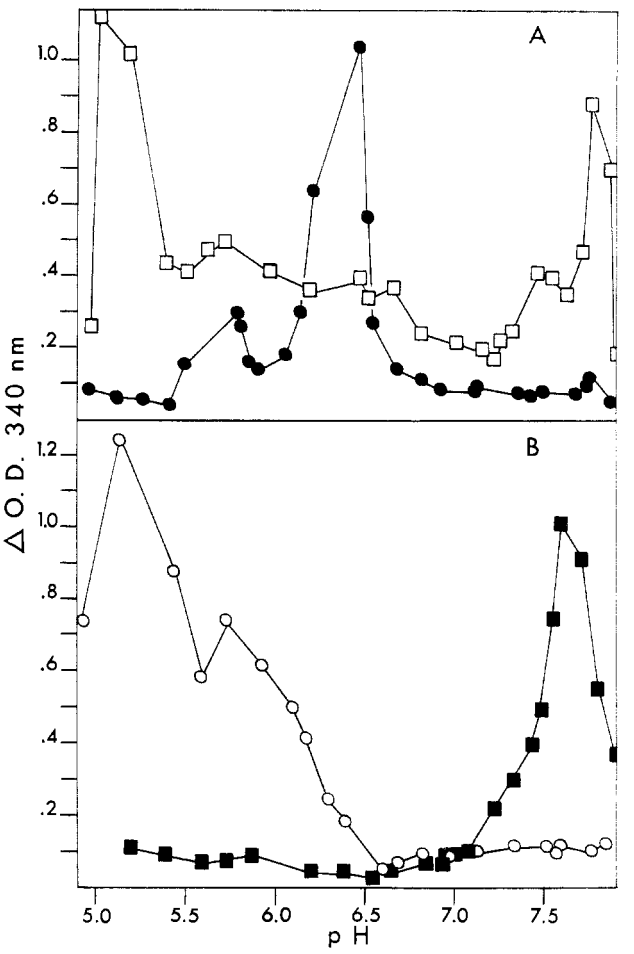

FIGURE 2: Profiles of pyruvate kinase activity obtained from isoelectric focused extracts and $(NH_4)_2SO_4$ fractions of kidney cortex. In part A the ● represent data obtained with extracts continuously held at 4°, while the □ represent data obtained in extracts incubated at 32° for 30 min at pH 6.8–6.9. Ninety-two per cent of the added activity was recovered for the unincubated sample and 81% for the incubated one. In the unincubated and incubated samples 18.0 and 18.2% of total recovered activity was found in the pH 5.7–5.8 peak, respectively. Incubation reduced the relative amount of enzyme found in the pH 6.2–7.0 range from 66.5 to 20.0% of the total, but increased the relative proportion of pH 5.1–5.2 enzyme by 18% and the pH 7.4–7.7 enzyme by 21% of the total recovered activity. Some unincubated preparations had relatively more 5.1 and 7.4–7.7 material. In part B, the ○ represent data obtained with a 40% saturated $(NH_4)_2SO_4$ precipitate of an unincubated kidney cortex extract, while the ■ represent data obtained from the supernatant from a 50% saturated $(NH_4)_2SO_4$ solution.

one band of activity was obtained from incubated extracts or nonincubated extracts from 3-day fasted animals. However, the apparent molecular weight (145,000) found for the incubated extracts was about midway between the two forms obtained from nonincubated samples. Conceivably the data truly represent samples with greater proportions of lower molecular weight enzyme in which the two bands have not been sufficiently separated.

The mean apparent molecular weight of the most rapidly sedimenting bands of activity utilizing all the acceptable data except those obtained after incubation was 174,000 (±5000 (SE); 10 determinations).

LIVER. Pyruvate kinase in the high-speed supernatant solution from liver homogenates continuously maintained in the cold also was activated by Fru-1,6-di-P (Table I). Thus neither the kidney cortex enzyme nor the liver enzyme became converted to the active form due to maintenance at 4°. However, incubation at 0° has been reported (Llorente *et al.*, 1970) to induce such a conversion. Incubation at 32° did not create a high $K_{0.5S}$ form as was the case for kidney cortex enzyme, but Fru-1,6-di-P activation was increased as previously observed by Bailey *et al.* (1968).

In the fasted animal the specific activity was more than halved (Table I) as has been reported by Tanaka *et al.* (1967), and Krebs and Eggleston (1965). Moreover, extracts from fasted animals show convex-up double-reciprocal plots and other evidence suggestive of the possible presence of two kinetic forms. Attempts to resolve the curve suggest that the majority of the enzyme had properties similar to that of the original extracts whereas the remainder had a somewhat higher $K_{0.5S}$ value (Table I).

Sedimentation data showed no evidence of heterogenity. Utilizing all the data obtained from extracts, a mean apparent molecular weight of 192,000 (±4500 (SE); 12 determinations) was obtained. This compares to a previously reported value of 208,000 obtained by sedimentation equilibrium (Tanaka *et al.*, 1967).

HEART. The high-speed supernatant solutions from heart muscle showed little or no Fru-1,6-di-P activation. The $K_{0.5S}$ value remained around 5.5×10^{-5} M for P-enolpyruvate under all conditions studied (Table I). These values are similar to the one of 7.5×10^{-5} M reported by Tanaka *et al.* (1967) for rat skeletal muscle. The sedimentation values also were not influenced by incubation. The mean apparent molecular

TABLE II: Isoelectric Values and Kinetic and Sedimentation Data Obtained from Electrofocused Kidney Cortex Extracts.

pI Value				Kinetic Properties					App Mol Wt × 10^{-3}		
				No Fru-1,6-di-P		5×10^{-5} M Fru-1,6-di-P					
Mean	±SE	No. of Detm	Remarks	$K_{0.5S}$[a]	n_H	Activation[b]	$K_{0.5S}$[a]	n_H	Mean	±SE	No. of Detm
5.13	0.020	10	Usually obtained after extracts have been incubated	6.5	1.2	2.4	6.5	1.2	115	7.9	4
5.45	0.024	4	Occasionally obtained from incubated extracts	3.3	1.0	1.3	3.3	1.0			
5.76	0.014	22	Always present but never a major form	1.8	1.1	2.6	0.75	1.0	183	9.8	2
6.41	0.022	20	Usually the major form obtained from unincubated extracts	1.5	1.2	3.5	0.60	1.0	199	3.8	4
6.77	0.026	14	Sometimes the major form from unincubated extracts	0.63	1.0	1.2	0.65	1.0	214	5.0	5
7.22	0.024	4	Occasionally found in trace quantities in incubated extracts								
7.50	0.056	8	Usually produced in small quantities upon incubation	16.0	1.4	5.1	16.0	1.0	104	5.6	6
or 7.44[c]	0.028	12									
7.70 or 7.67[c]	0.014	21	Always obtained from incubated extracts	12.0	1.0	2.0	12.0	1.0	106	8.9	3

[a, b] As in Table I. [c] Upper number is the mean value based upon the relative position of the two activity bands with the highest pI values. Lower number is the mean value obtained when these two bands are identified on the basis of the n_H value. Kinetic data were selected on the basis of the n_H value.

weight obtained from enzyme in all the heart preparations studied was 208,000 (±3000 (SE); 6 determinations). This compares to a value of 250,000 reported for rat skeletal muscle by sedimentation equilibration (Tanaka *et al.*, 1967). A statistical comparison by the "t" test of values obtained for heart enzyme against those for enzymes from liver or kidney show both to differ significantly from the heart enzyme, having *P* values of less than 0.01. A test of the possible statistical significance of the difference between the values of liver and kidney enzymes yielded a *P* value of 0.02.

Solubility of Enzymes in $(NH_4)_2SO_4$. Figure 1 shows the majority of pyruvate kinase present in extracts of kidney cortex, liver or heart is precipitated at different concentrations of $(NH_4)_2SO_4$. Eighty-seven per cent of the activity obtained from liver extracts was precipitated between 30 and 40% saturation, while heart muscle enzyme was precipitated maximally at 60–70% saturation. These findings are consistent with those reported by Tanaka *et al.* (1967) and Taylor *et al.* (1969) for rat liver and skeletal muscle enzyme, respectively. Kidney cortex extracts had the majority of activity precipitated between 50 and 60% saturation. The nonsymmetrical nature of the curves obtained for the kidney cortex and liver isoenzymes suggests that each is a mixture containing small quantities of the other.

Isoelectric Focusing Patterns. KIDNEY CORTEX. Typical isoelectric focusing patterns obtained with the high-speed supernatant fractions obtained from homogenates of kidney cortex are illustrated in Figure 2A. When the enzyme sample was maintained at 4° from the time of sacrifice, the majority of the enzyme was found to have a mean pI value of either 6.41 or 6.77[2] (Table II). Generally the form with a lower pI value predominated as in Figure 2A. A third band of activity was isolated with a mean pH of 5.76. When tissue extracts were incubated in the presence or absence of added Fru-1,6-di-Pase or Fru-1,6-di-P at 32° for 30 min at pH values ranging from 6.8 to 7.8, the pH 6.41 or 6.77 enzyme became less predominant and peaks with mean pI values of 5.13 and 7.70 became more predominant. Smaller proportions of enzyme with mean pI values of 7.22 and 7.4–7.5 also became evident (Figure 2A and Table II). The relative proportion of pH 5.13 to 7.70 material was quite variable. In some experiments roughly equal quantities of the two were produced, in others one of the two predominated. In a few experiments pH 5.13 material was not produced but instead enzyme was obtained at pH 5.45. In these experiments there appeared to be smaller quantities of a precipitate which was prone to form at about pH 5.2.

In order to test the possibility that the changes induced by incubation were due to proteolytic activity, homogenization and incubation were performed in the presence of 1×10^{-3} M phenylmethylsulfonyl fluoride, a general protease inhibitor

[2] When referring to the collective data concerning a particular form, the pI values are expressed as the mean value to the nearest hundredth. When an individual run (or at most a few runs) is referred to the actual determined pI value is reported to the nearest tenth of a pH unit.

TABLE III: Interconversions Obtained with Kidney Cortex Isoenzymes.

Initial pI Value	Conditions Employed[a]	% of Recovd Act. with a pI Value of[d] 5.13	5.43	5.76	6.1	6.41	6.77	7.22	7.44	7.67
7.67	Incubated									100
7.67	Incubated with Fru-1,6-di-P						47.2	6.5	2.5	38.7
6.41	Incubated at pH 7.2 with Fru-1,6-di-Pase		11.0			12.0	2.0		10.0	62.0
6.41	Incubated with Fru-1,6-di-P		15.0			11.5	63.7			
5.76	Incubated at pH 7.8 with Fru-1,6-di-Pase			33.0	60.0[b]					
5.76	Incubated with Fru-1,6-di-P			90[c]	5.0					
5.45	Incubated at pH 6.9 with Fru-1,6-di-P		11.0	36.0		8.3	5.2			23.0
5.13	No manipulations		8.7			38.0				32.0
5.13	Incubated at pH 7.1 with Fru-1,6-di-P		8.9	34.0		4.5	1.9	1.5		26.6

[a] Incubated at 32°, 30 min. When present, the concentration of Fru-1,6-di-P was 7 μM. Additionally the column was made to 1 μM with respect to Fru-1,6-di-P. If a pH value is not indicated incubation was performed at the isoelectric pH. [b] Probably represents the mean pH value of two bands with similar pI values. [c] pI value 5.6. [d] For convenience in relating these data to those of Table II, the mean pI values are indicated in the heading. The actual pH values or actual mean (two to three runs) are indicated in text as reported to the nearest 0.1 unit.

(Fahrney and Gold, 1963); 1×10^{-3} M ϵ-amino-*n*-caproic acid, a competitive inhibitor of carboxypeptidase B (Ambler, 1967); and 1×10^{-3} M iodoacetate (added in the absence of 2-mercaptoethanol), an extremely potent inhibitor of cathepsins B (Greenbaum and Fruton, 1957) and C (Fruton and Mycek, 1956). No significant changes were observed in the presence of any of these proteolytic inhibitors. The presence or absence of 2-mercaptoethanol also did not alter the pattern of change induced by incubation.

Enzyme corresponding to each major isoelectric band was subjected to kinetic and sedimentation analysis (Table II). Enzyme with pI values of about 6.4 was Fru-1,6-di-P activated, while enzyme with pI values around 6.7 was not. Similarly pH 7.4–7.8 enzyme tended to fall into two groups, the form with the lower pI value again being more activated by Fru-1,6-di-P. Since in this case, however, overlap did occur, the data of Table II are presented with respect to the two ways of discriminating, namely: on the basis of their relative position (the major peak with the highest pI value gave a mean value of 7.70, the smaller peak or shoulder with a lower pI value gave a mean value of 7.50); or on the basis of the effect of Fru-1,6-di-P on the n_H value[3] (the respective mean pI values being 7.67 and 7.44).

The two higher and the lowest pI forms had approximately half the apparent molecular weight and a $K_{0.5S}$ value for P-enolpyruvate (when the enzyme was activated) of an order of magnitude higher than did the pH 5.76, 6.41, or 6.77 forms of the enzyme. Only limited data were obtained for the pH 5.45 and 7.22 forms since they were obtained in too low a yield or too irregularly. Kinetic data suggest the pH 5.45 enzyme consists predominantly of a high $K_{0.5S}$ form (estimated value 3×10^{-4} M).

Table III summarizes data obtained as the result of attempts to interconvert the various pI forms. In all cases described, the initial enzyme was characterized by kinetic and sedimentation properties as well as pI value prior to refocusing. It was possible to convert a pH 7.8 enzyme to a pH 6.7 enzyme by incubating with Fru-1,6-di-P and then electrofocusing. Only trace amounts of enzyme was converted if the high pI material were simply mixed with this hexose phosphate but not incubated. In three experiments conducted, no enzyme with a pI less than 6.7 was formed. Kinetic and sedimentation data showed this newly generated pH 6.77 material to be indistinguishable from that obtained from fresh kidney cortex extracts.

Incubation of a pH 6.4 enzyme with Fru-1,6-di-P generated pH 6.8 enzyme, which again had the kinetic and sedimentation properties expected of the pH 6.77 enzyme. Significant amounts of enzyme with a pI value of 5.4 were also generated. When 6.4 material was incubated with Fru-1,6-di-Pase, the majority of the enzyme formed had a pI value of 7.8. This was shown to have a high $K_{0.5S}$ value and a low sedimentation rate, similar to the pH 7.70 band obtained from incubated extracts. A small proportion of the enzyme was obtained having a pI value of 5.5. Kinetic analysis of the limited 5.4 or 5.5 enzyme available showed it to have a $K_{0.5S}$ value greater than 10^{-4} M P-enolpyruvate and not to be Fru-1,6-di-P activated.

Incubation of the 5.8 material with Fru-1,6-di-Pase generated a broad band of enzyme activity with a mean pI value of about 6.1. Incubation of an enzyme with a pI value of 5.8

[3] That Fru-1,6-di-P lowered the n_H value, and therefore eliminated the homotropic activation effect, but did not lower the $K_{0.5S}$ value was a mathematical consequence of the fact that Hill plots, drawn for enzyme assayed in the presence and absence of Fru-1,6-di-P, intersected at the $K_{0.5S}$ value. Functionally this appears to signify that the catalytic $K_{0.5S}$ value for P-enolpyruvate is so high that homotropic activation is complete prior to achieving 0.5 V_{max}. This would imply that P-enolpyruvate binds both at a catalytic and allosteric site.

TABLE IV: Isoelectric Values and Kinetic and Sedimentation Data Obtained from Electrofocused Liver and Heart Extracts.

Tissue	pI Value Mean	±SE	No. of Detm	Remarks	Kinetic Properties: No Fru-1,6-di-P $K_{0.5S}$[a]	n_H	5 × 10⁻⁵ M Fru-1,6-di-P Activation[b]	$K_{0.5S}$[a]	n_H	App Mol Wt ×10⁻³ Mean	±SE	No. of Detm
Liver	5.46	0.010	4	Obtained from unincubated extracts or extracts incubated at pH values below 7.0	0.69	1.0	1.6	0.67	1.0	190	6.6	3
	5.73	0.056	4	Obtained from unincubated extracts of 3-day fasted rats or extracts from fed animals incubated above pH 7.0	1.6	1.2	2.0	0.71	1.0	172		1
	6.48	0.030	5	Obtained from unincubated extracts	0.95	1.2	2.0	0.45	1.0	194		1
	6.71	0.010	2	Present in trace amounts								
	7.26	0.010	2									
	7.59	0.080	2									
Heart	6.56	0.063	5	Present in trace amounts	0.52	1.0	1.0	0.52	1.0	240		1
	7.23	0.030	5	Always present in moderate amounts	0.40	1.0	1.0	0.40	1.0	234		1
	7.48	0.028	3	Major peak in unincubated or acid incubated extracts	0.52	1.0	1.0	0.52	1.0	228		1
	7.55	0.035[c]	2	Major peak in extracts incubated above pH 7.0 with Fru-1,6-di-Pase	0.68	1.0	1.0	0.58	1.0	209	8.4	2

[a, b] As in Table I. [c] Data include reelectrofocused sample.

with Fru-1,6-di-P lowered the p*I* value to 5.6 (Table III). Due to the limited quantities of this enzyme initially available and due to the losses in handling, recovered activities were too low to perform either kinetic or sedimentation studies.

Re-electrofocusing pH 5.2 material generated pH 5.8, 6.4, 6.8, and 7.8 material (Table III). Identical results were obtained with a band which had a p*I* value of 5.4. The 7.8 material was shown to have the same $K_{0.5S}$ value and molecular weight as did the 7.70 enzyme from extracts. The newly generated pH 5.8 material had a $K_{0.5S}$ value of about 7.0×10^{-5} M, while the residual pH 5.4 material had a $K_{0.5S}$ value of about 4×10^{-4} M.

Attempts were also made to characterize the isoelectric isoenzymes in terms of their solubility in $(NH_4)_2SO_4$. These data were relatively variable, perhaps due to the presence of glycerol or ampholines and because of relatively small volumes available. It was clear, however, that the pH 5.13 and 5.76 peaks were the least soluble, being about 50% precipitated at 40% saturation. The remaining major peaks were about 20–30% precipitated at 40% saturation. When the unincubated supernatant of the cortex homogenate was subjected to $(NH_4)_2SO_4$ precipitation prior to isoelectric focusing, results similar to that shown in Figure 2B were obtained. The 0–40% precipitate contained, almost exclusively, pH 5.13 and 5.76 material, whereas the material which was still soluble at 50% saturation consisted almost exclusively of pH 7.70 material. Experiments were also performed in which no material was excluded. That is, a 40 or 45% saturation cut was obtained and both the precipitate and supernatant were subjected to electrofocusing. Although there was more cross contamination, similar data were obtained, and more importantly, most of the pH 6.2–6.8 material had disappeared. Thus $(NH_4)_2SO_4$ precipitation in the cold induced a change similar to that induced by incubation at 32°.

LIVER. Figure 3 shows the isoelectric pattern obtained from liver extracts. Incubated and unincubated extracts gave similar patterns, having almost all the activity in a band with a mean p*I* value of 5.46, and small quantities of enzyme with peaks at mean pH's of 6.48 or 6.71, 7.26, and 7.60, the latter two being present in extremely small amounts and only in incubated extracts. Equally complex isoelectric patterns have previously been reported for liver extracts from several mammalian species (Criss, 1969; Hess and Kutzbach, 1971; Yanagi *et al.*, 1971).

Extracts incubated at pH values greater than 7 or unincubated extracts from fasted animals had the majority of the pH 5.46 enzyme replaced by enzyme having a p*I* value of 5.73. Kinetically the 5.73 material differed from the pH 5.46 enzyme in that only the former showed heterotropic activation (Table IV).

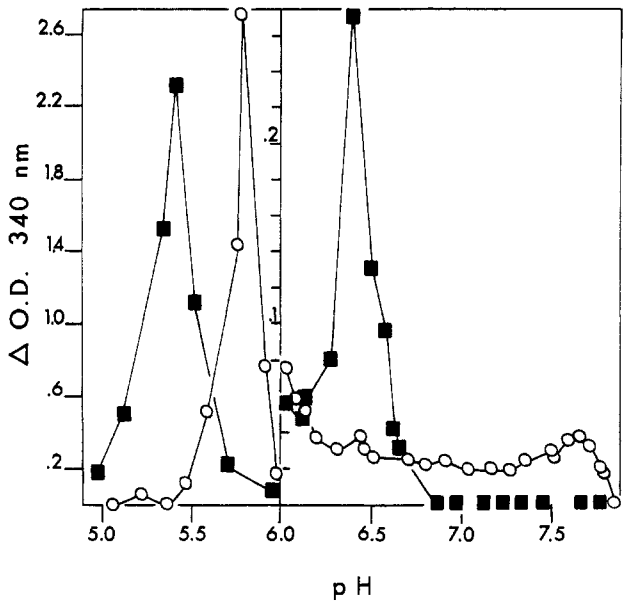

FIGURE 3: Profile of pyruvate kinase activity obtained after liver extracts were subjected to isoelectric focusing. The ■ represent data obtained from an unincubated sample, while the ○ represent data obtained from a sample incubated in the presence of Fru-1,6-di-Pase for 30 min at 32° at pH 7.0. Essentially all activity added was recovered. In both incubated and unincubated extracts 90.6% of the total recovered activity was found in the major peak. In the unincubated preparation almost all the remaining activity, 9.3%, was found in a peak with a p*I* value of 6.41. (Note the scale change at pH 6.0.) Incubation reduced this peak and increased activity at higher p*I* values. This pH 6.41 peak also was reduced when incubated at pH values below pH 7.0, whereas the shift in the main activity peak was only observed if the incubation was conducted at pH 7.0 or greater. No attempt was made to see if the shift in p*I* values was actually influenced by the exogenous Fru-1,6-di-Pase.

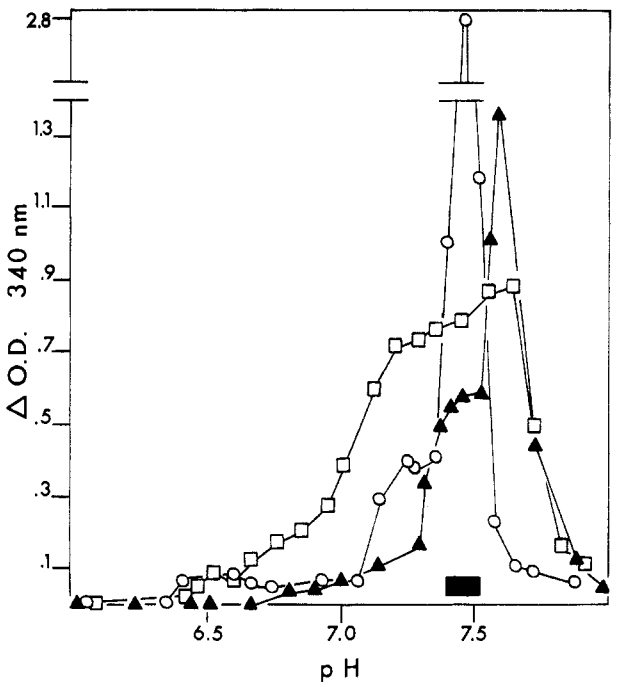

FIGURE 4: Profile of pyruvate kinase activity obtained by electrofocusing rat heart extracts. (○) Data obtained from an unincubated sample; (□) data obtained from a sample incubated 30 min at 32° at pH 7.7. The total activity was normalized so that these two experiments are equivalent. (▲) Data obtained when the three tubes from the unincubated sample in the pH range 7.4–7.5 (as indicated by the bar) were collected and then incubated with added Fru-1,6-di-Pase. All added enzyme was recovered after electrofocusing the unincubated sample, while 80% of the incubated sample was recovered and 60% of the reelectrofocused material. No activity was obtained at pH values between 4.8 and 6.3.

The pH of an enzyme sample having a p*I* value of 5.7 was adjusted to pH 7.0 and divided into two aliquots. To one Fru-1,6-di-Pase was added, to the other Fru-1,6-di-P. Both were incubated at 32° for 30 min and reelectrofocused. Some 61% of the recovered enzyme incubated with Fru-1,6-di-P (as in Table III) had a p*I* value of 5.5, while 35% had a p*I* value of 7.5. Three peaks of activity were obtained using the enzyme incubated with Fru-1,6-di-Pase. About 53% had a p*I* value of 5.8, 21% yielded a broad activity band with a mean p*I* value of 6.1 and 21% had a p*I* value of 7.4. The apparent molecular weights of the pH 7.4–7.5 enzymes were 219,500 and 230,000 and the $K_{0.5S}$ values for P-enolpyruvate 5.0×10^{-5} and 4.0×10^{-5}, for the Fru-1,6-di-P and Fru-1,6-di-Pase incubated samples, respectively. Kinetic parameters were not markedly affected by Fru-1,6-di-P in the assay mix.

The minor liver peak with a mean p*I* value of 6.48 behaved similarly to the major pH 6.41 kidney cortex enzyme with respect to kinetics, sedimentation, and stability (Table IV and Figure 3).

HEART. The isoelectric focusing pattern obtained using extracts of heart are shown in Figure 4. Unincubated samples showed a major peak at a mean pH of 7.48 with a secondary peak or shoulder at pH 7.23. A small fraction of enzyme was obtained with a mean p*I* value of 6.56 (Table IV). When such extracts were incubated with Fru-1,6-di-Pase at 32° for 30 min under mildly alkaline conditions, the pH 7.48 enzyme was reduced in activity and enzyme with higher and lower p*I* values was produced. Likewise when the tubes representing the sharp activity peak in the unincubated sample (pH range 7.4–7.5) were reincubated the major peak was shifted to 7.58. Again some enzyme with a lower p*I* value may also have been generated (Figure 4). As shown in Table IV all of these activity peaks have low $K_{0.5S}$ values and relatively high sedimentation rates.

The p*I* values obtained for rat heart pyruvate kinase differ markedly from the pH 6.75 value previously reported for rat skeletal muscle enzyme (Criss, 1969). On the other hand, the activity profile is similar to that reported for mouse skeletal muscle (Yanagi *et al.*, 1971). In order to test the possibility that a difference existed between heart and skeletal muscle enzyme, unincubated skeletal muscle extracts were also electrofocused. A symmetrical peak with a p*I* value of 7.5 was obtained.

Discussion

Kidney Cortex. A specific goal of these studies was to determine the nature of kidney cortex pyruvate kinase relative to the two previously well described isoenzymes. The majority of activity in nonincubated kidney cortex extracts had a p*I* value which differed from the major enzymes of liver or heart by more than 0.6 of a pH unit. This enzyme also had distinctive solubility properties and unlike the heart or liver enzyme it tended to dissociate into subunits when incubated. These data strongly support the concept that the major enzyme of kidney cortex is a distinct molecular species.

That the unique p*I* value of the kidney cortex enzyme is fundamental and not a function of nonspecific interaction

with substances in the extract is supported by the facts that the enzyme may be partially purified in either the higher or lower p*I* forms, then be reassociated and reisolated as enzyme with the initial p*I* value and sedimentation and kinetic properties (Table III).

Kinetically the high molecular weight kidney cortex enzymes were very different than any of the heart enzymes, but similar to the major liver enzymes. More detailed preliminary kinetic studies on the pH 6.41 kidney isoenzyme dialyzed free of ampholines suggest it may differ subtly from the liver enzyme. In particular, the kidney enzyme appears to be more sensitive to alanine inhibition. The half-inhibition concentration at pH 7.5 for the kidney cortex enzyme is about 5×10^{-5} M; this compares to values of about 0.7×10^{-3} to 1.5×10^{-3} M reported for liver enzyme (Seubert and Schoner, 1971).

Jiménez de Asúa *et al.* (1971a) found about 80% of the pyruvate kinase in kidney cortex extracts to be eluted from DEAE columns with the void volume, in contrast to the remainder and to the major liver isoenzyme which were eluted at about 100 mM KCl. When the pH 6.41 peak was dialyzed against the buffer used by these authors, and subjected to DEAE chromatography under their conditions, it too was eluted at about the void volume. Although the enzyme was in the high molecular weight, low $K_{0.5S}$ Fru-1,6-di-P-activatable form prior to dialysis, the enzyme recovered in the eluate had a $K_{0.5S}$ value for P-enolpyruvate of 10^{-3} M and little or no Fru-1,6-di-P activation. These are indeed the kinetic properties ascribed by Jiménez de Asúa *et al.* to the "unique" kidney cortex enzyme and of the subunits isolated by electrofocusing incubated enzyme (Table II).

The remaining third of the activity obtained from most unincubated homogenates appeared largely as enzyme with a mean p*I* value of 5.76. This band of activity appeared to be identical with the major liver isoenzyme as indicated by the p*I* value, its solubility properties, the apparent inability to interconvert pH 5.76 and 6.41 or 6.77 enzyme, and the similar products obtained when partially purified enzyme from liver or kidney was reelectrofocused (Tables II and III and Results section). This interpretation also is consonant with the chromatographic data of Jiménez de Asúa *et al.* (1971a) and the electrophoretic data of Susor and Rutter (1971). This pH 5.76 band of activity also was subjected to DEAE chromatography. In contrast to the pH 6.41 enzyme and as would be expected for liver enzyme, it was eluted at a relatively high ionic strength.

That the major kidney cortex enzyme readily broke down into enzyme with about half the original molecular weight, but with p*I* values similar to the liver enzyme, on one hand, and the muscle enzyme, on the other, suggested the enzyme was a hybrid of liver and muscle subunits. The different kinetic properties would be ascribed to the changed degree of polymerization. However, the fact that the low p*I* enzyme could be converted to high p*I* enzyme, while high p*I* enzyme could be converted back to the original polymer (Table III), appeared to rule out this possibility. That low p*I* material could form the highest p*I* enzyme but the reverse was not true suggests that the former differed from pH 7.70 enzyme in that it had a relatively low molecular weight component associated with it. That the production of neither of these apparent subunits was decreased by inhibitors of proteolytic enzymes and that the "subunits" could be reconverted to the original polymeric form indicate that changes in the primary structure were not involved. Moreover, to the degree that these reactions were reversible, deamidation or deacylation could not account for the changes. Finally since deamidation should lower the p*I* value, one would not expect the apparently irreversible conversion of the pH 5.13 or 5.43 enzyme to the 7.70 form to involve loss of amide nitrogen.

Although the data suggest the higher molecular weight pH 6.41–6.77 enzyme is the major *in vivo* form, the appearance of significant amounts of lower molecular weight enzyme in fresh preparations (Table I) is reminiscent of the situation obtained with fresh human erythrocytes (Ibsen *et al.*, 1971), and suggests that quaternary interchange is a potential control mechanism. The high $K_{0.5S}$ values of the lower molecular weight forms would make them inactive under physiological conditions.

The low and high molecular weight enzymes appeared to exist in sets of two (*i.e.*, pH 5.13 and 5.45, pH 6.41 and 6.77, and pH 7.50 and 7.70). It is postulated (for at least the two higher p*I* sets) that these represent R,T conformational pairs. In both cases only enzyme with a lower p*I* value shows either hetero-[4] or homotropic activation. Moreover, incubation with the heterotropic activator appears to convert the lower p*I* member of the pair to the higher p*I* form. No such relationship has emerged for the lowest p*I* pair. However, these low p*I* enzymes are more difficult to investigate due to the high glycerol content of these fractions, the tendency for a precipitate to form at these pH values, and the tendency for the 5.45 enzyme to appear irregularly. Moreover, in several cases evidence has been adduced to suggest that both these enzymes could contain some higher molecular weight enzyme, probably liver pH 5.46 enzyme. This could account for the ability of the pH 5.13 and 5.45 enzyme to form some pH 5.76 enzyme (Table III) upon reelectrofocusing. On the other hand, these data could imply that the two isoenzymes, although not readily interconvertible, shared a basic primary structure.

Liver. Approximately 90% of the total activity of liver pyruvate kinase was found in a relatively homogeneous peak at a mean pH of 5.46 or 5.73. These enzymes once again bore the characteristics of an R,T conformational pair, in which the higher p*I* enzyme was the less active form. Enzyme from fed animals invariable showed a predominance of pH 5.46 enzyme which could be converted to the pH 5.73 form by incubation at alkaline pH values. This is consonant with the concept that destruction of Fru-1,6,di-P permitted the enzyme to be transformed to the T conformation since Fru-1,6-di-Pase has an alkaline pH optimum. This conclusion is also consistent with the observation that only the higher p*I* form was able to undergo hetero- or homotropic activation. Hess and Kutzbach (1971) arrived at a similar conclusion using purified pig liver enzyme and radioactive Fru-1,6-di-P. They found that only the lower p*I* form bound the activator.

It is significant that incubation at pH values less than neutral did not induce a change in conformation nor did incubation ever cause apparent dimer to be formed. This represents a distinct difference from the kidney cortex enzyme.

Starvation also induced the same conformational change as did incubation at alkaline pH values. This demonstrates that this change could be of true physiological value, but it does not indicate which of the several mechanisms available for the conversion is the most significant.

Since the primary goal of this study was to evaluate the relative nature of the kidney cortex enzyme, the interconvertibility of the liver enzyme was not as extensively studied.

[4] That the apparent T conformers of electrofocused kidney cortex and liver enzyme are in a more active form than in fresh homogenates may represent partial activation by H^+ (Rozengurt *et al.*, 1969).

However, it was shown that Fru-1,6-di-P converted the pH 5.73 enzyme to the lower p*I* form as would be expected of an R,T conformational pair. What was unexpected, however, was the fact that incubation of the partially purified pH 5.73 peak generated significant amounts of pH 7.44 enzyme. This derived enzyme had sedimentation and kinetic properties similar to muscle enzyme and distinctly different from the derived kidney enzyme with a similar p*I* value. If liver enzyme can be converted into muscle enzyme then they do not represent the product of distinct cistrons. Since, however, these enzymes are obviously different and not readily interconvertible, it would seem probable that the enzyme has been altered in the liver cell by a noncovalent but relatively stable addition or that the liver enzyme was a hybrid containing muscle type subunits.

The remaining 10% of activity obtained in nonincubated liver extracts had a p*I* value, kinetic and sedimentation characteristics similar to the kidney isoenzyme. Moreover, incubation even at pH values below 7.0 caused this peak to disappear and be replaced by higher p*I* enzyme, as was the case for the primary kidney cortex enzyme. These data lead one to conclude that kidney cortex enzyme is also present in extracts of liver. This conclusion is also consonant with the solubility data (Figure 1). This conclusion is at variance, however, with the generally accepted concept that liver pyruvate kinase is a mixture of type L and M enzyme as has been reported for rat by Tanaka *et al.* (1967), and Criss (1969), for mouse by Yanagi *et al.* (1971) and for humans by Bigley *et al.* (1968). On the other hand, Hess and Kutzbach (1971) find no noninterconvertible variants of pyruvate kinase in pig or rat liver, while Susor and Rutter (1968, 1971) and Farron *et al.* (1972) find a minor band with electrophoretic mobilites more similar to the major rat kidney enzyme than to rat muscle enzyme, and Jiménez de Asua *et al.* (1971b) find type "M" enzyme of liver to have different kinetic properties than type "M" enzyme from muscle. These inconsistencies could once again suggest that the various molecularly distinct isoenzymes of pyruvate kinase differ not because they are the product of different cistrons, but rather because of a subsequent alteration which is subject to some variation depending upon conditions.

Heart Muscle Enzyme. In nonincubated heart or skeletal muscle extracts the vast majority of the enzyme had a p*I* value of 7.48 (Figure 4, text). The heart enzyme also showed a shoulder of activity having a mean p*I* value of 7.23 while a trace of activity was found at pH 6.56. Incubation of heart extracts or partially purified heart enzyme at alkaline pH values decreased the pH 7.48 enzyme and created a pH 7.55 enzyme. However, these forms had similar kinetic and sedimentation properties. The changes responsible for the different p*I* could possibly involve deamidation or deacylation reactions.

The decrease in specific activity obtained in heart extracts upon starvation was unexpected. Tanaka *et al.* (1967) had reported that skeletal muscle enzyme activity was not affected by diet or hormones. Thus, if type M enzyme was produced by a unique cistron one would conclude that the response of this cistron varied from tissue to tissue, or alternately these data could again suggest that the different molecular species of mammalian pyruvate kinase result from alterations subsequent to polymerization.

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