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Identity of Isoenzyme 1 of Histidine–Pyruvate Aminotransferase with Serine–Pyruvate Aminotransferase

By TOMOO NOGUCHI, ETSUO OKUNO and RYO KIDO
Department of Biochemistry, Wakayama Medical College, Wakayama 640, *Japan*

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After glucagon injection, rats showed virtually identical percentage increases in hepatic histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities, both in the mitochondria and in the cytosol. Histidine–pyruvate aminotransferase isoenzyme 1, with pI 8.0, was purified to homogeneity from the mitochondrial fraction of liver from glucagon-injected rats. The purified enzyme catalysed transamination between a number of amino acids and pyruvate or phenylpyruvate. For transamination with pyruvate, the activity with serine reached a constant ratio to that with histidine during purification, which was unchanged by a variety of treatments of the purified enzyme. Serine was found to act as a competitive inhibitor of histidine transamination, and histidine of serine transamination. These results suggest that histidine–pyruvate aminotransferase isoenzyme 1 is identical with serine–pyruvate aminotransferase. The enzyme is probably composed of two identical subunits with mol.wt. approx. 38000. The absorbance maximum at 410nm and the inhibition by carbonyl reagents strongly indicate the presence of pyridoxal phosphate.

Histidine–pyruvate aminotransferase (EC 2.6.1.–) and serine–pyruvate aminotransferase (EC 2.6.1.51) have been studied as distinct enzymes. Histidine–pyruvate aminotransferase is present in both the mitochondrial and supernatant fractions of rat liver (Spolter & Baldridge, 1964; Budillon *et al.*, 1971) and both activities are increased by the injection of glucagon, possibly by a cyclic-AMP-dependent mechanism (Morris *et al.*, 1973). We have reported that rat liver contains two forms of histidine–pyruvate aminotransferase (Noguchi *et al.*, 1976*a*,*b*): one, designated isoenzyme 1 (pI 8.0), is present only in the liver and is induced by the injection of glucagon; the other, designated isoenzyme 2 (pI 5.2), is found in all tissues tested and is not affected by glucagon.

It has also been documented that serine–pyruvate aminotransferase activity is present in rat liver and is increased by the injection of glucagon (Rowsell *et al.*, 1969, 1972, 1973; Sallach *et al.*, 1972). The present report describes the identity of histidine–pyruvate aminotransferase isoenzyme 1, with pI 8.0, with serine–pyruvate aminotransferase.

Experimental

Materials

Spinach D-glycerate dehydrogenase (D-glycerate–NAD^+ oxidoreductase, EC 1.1.1.29) and sodium phenylpyruvate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-Amino acids were from Tanabe Amino Acid Foundation, Osaka, Japan. KCN, hydroxylamine hydrochloride and semicarbazide were obtained from Nakarai Chemicals, Kyoto, Japan. Other chemicals were obtained as stated previously (Noguchi *et al.*, 1976*a*).

Methods

Transamination between aromatic L-amino acids (histidine, tyrosine, phenylalanine, tryptophan and 5-hydroxytryptophan) and α-oxo acids (pyruvate, 2-oxoglutarate and oxaloacetate) was determined as described by Noguchi *et al.* (1976*a*). This assay was based on the arsenate-catalysed formation of aromatic 2-oxo acid–enol-borate complexes, which show characteristic absorption spectra in the 300nm region. The assay mixtures (0.8ml) contained, unless specified otherwise, 20mM-aromatic amino acid, 20mM-α-oxo acid, 0.2M-Tris/HCl, pH 9.0, and enzyme preparation. Tyrosine was used at 6.5mM because of its low solubility.

Transamination between L-serine and α-oxo acids (pyruvate, 2-oxoglutarate, oxaloacetate and phenylpyruvate) was determined as described by Sallach *et al.* (1972). The reaction mixtures (0.4ml) contained 20mM-serine, 20mM-α-oxo acid, 0.2M-Tris/HCl, pH 9.0, and enzyme preparation. The amount of hydroxypyruvate formed after incubation at 37°C was determined with NADH and spinach leaf D-glycerate dehydrogenase.

Transamination between various aliphatic L-amino acids and phenylpyruvate was determined by the method of Kupchik & Knox (1970). The reaction

mixtures (0.8ml) contained 20mM amino acid, 1.4mM-phenylpyruvate, 0.2M-Tris/HCl, pH9.0, and enzyme preparation. The disappearance of the enol–borate complex of phenylpyruvate was continuously followed at 300nm.

A unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of product/h at 37°C.

Protein was determined by the biuret method (Gornall *et al.*, 1949) in crude extracts or by the method of Lowry *et al.* (1951) in purified preparations. Bovine serum albumin was used in preparing a standard curve.

Subcellular fractionation of rat liver, polyacrylamide-disc-gel electrophoresis, isoelectric focusing and determination of approximate molecular weight by sucrose-density-gradient centrifugation and Sephadex G-150 gel filtration were carried out as described by Noguchi *et al.* (1976*a*).

Polyacrylamide-disc-gel electrophoresis in sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969). The following marker proteins were used for the estimation of the subunit molecular weight of the purified enzyme: bovine serum albumin (mol.wt. 67000), ovalbumin (43000), alcohol dehydrogenase (37000), carboxypeptidase A (34600) and cytochrome *c* (12380).

Results

Subcellular distribution of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase

Table 1 shows the distribution of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities in the subcellular fractions of rat liver. Both enzyme activities were similarly distributed and mainly found in the mitochondrial (heavy and light mitochondria) and supernatant fractions. Snell (1975) reported that only 6% of the serine–pyruvate aminotransferase of rat liver was recovered in the cytosol, and the 93% particulate enzyme appeared to be largely mitochondrial. The aminotransferase in the cytosol could have been derived from disrupted mitochondria, since glutamate dehydrogenase (L-glutamate–NAD^+ oxidoreductase, EC 1.4.1.2), the mitochondrial marker enzyme, was found in the cytosol to about the same extent. In the present study, monoamine oxidase [monoamine–oxygen oxidoreductase (deaminating), EC 1.4.3.4] was used as the mitochondrial marker enzyme. Only 1.4% of this enzyme activity was recovered in the cytosol and the remainder in the particulate fractions, definitely suggesting the presence of serine–pyruvate aminotransferase in the cytosol. The distribution of monoamine oxidase activity corresponded well to the results previously reported by Noguchi *et al.* (1976*a*). Compared with control rats, liver from glucagon-treated rats showed

Table 1. *Intracellular distribution of hepatic histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase from control and glucagon-injected rats*

Rats were housed in wire-bottomed cages and maintained at about 20°C in a room with a 12h light/12h dark cycle. Food and water were available *ad libitum*. Some were injected subcutaneously with glucagon suspended in 0.15% KCl (0.35 mg, every 8h for 2 days). Control rats received 0.15% KCl every 12h for 2 days. Rats were decapitated with a guillotine, and the livers removed. Subcellular fractionation of the liver and enzyme assays were carried out as described in the text.

Fraction	Protein (mg/g wet wt. of tissue)	Histidine–pyruvate aminotransferase				Serine–pyruvate aminotransferase			
		Specific activity (units/mg)		Total activity (%)		Specific activity (units/mg)		Total activity (%)	
		−Glucagon	+Glucagon	−Glucagon	+Glucagon	−Glucagon	+Glucagon	−Glucagon	+Glucagon
Homogenate	196	0.18	4.5	100	100	0.051	1.5	100	100
Nuclei	30.2	0.13	4.1	11.1	14.0	0.037	0.91	11.2	9.3
Heavy mitochondria	36.8	0.40	11.4	41.7	47.6	0.120	3.8	44.2	47.6
Light mitochondria	20.5	0.38	11.7	22.1	27.2	0.110	3.6	22.6	25.1
Microsomal fraction	31.9	0.02	0.04	1.8	0.1	0.007	0.009	2.2	0.1
Supernatant fraction	81.5	0.07	0.29	16.2	2.7	0.02	0.07	16.3	1.9
Recovery	200.9			92.9	91.6			96.5	84.0

virtually identical increases in histidine- and serine–pyruvate aminotransferase activities, both in the mitochondrial and in the supernatant fraction. The mitochondrial and supernatant activities were increased about 30-fold and 4-fold respectively. When the mitochondrial extracts and the supernatant fractions from livers of both control and glucagon-injected rats were subjected to isoelectric focusing on a pH3.5–10 Ampholine gradient as previously described (Noguchi *et al.*, 1976*a*), two histidine–pyruvate aminotransferase activity peaks, with pI8.0 and pI5.2 respectively, were obtained in each case. The enzyme with pI8.0 has been designated as isoenzyme 1 and that with pI5.2 as isoenzyme 2 (Noguchi *et al.*, 1976*a*). Isoenzyme 1 showed serine–pyruvate aminotransferase activity, but isoenzyme 2 did not. For isoenzyme 1, the activity with histidine was about 3.6 times that with serine in transamination with pyruvate. In comparison with control rat liver, isoenzyme 1 activity of glucagon-injected rats showed virtually identical percentage increases in total histidine–pyruvate and serine–pyruvate aminotransferase activity both in the mitochondrial fraction and in the supernatant fraction. The mitochondrial and supernatant isoenzyme 1 activities were elevated by about 38-fold and 5-fold respectively. However, isoenzyme 2 activities were not affected by glucagon injection in either subcellular fraction. These data suggest that isoenzyme 1 is identical with serine–pyruvate aminotransferase.

Purification of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase

Purification of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase from the mitochondrial fraction of liver from glucagon-injected rats was carried out to obtain further evidence on the identity of these enzymes. All manipulations were carried out at 0–4°C. Glass-distilled water and potassium phosphate buffer, pH7.5, were used throughout. At each stage in the purification aminotransferase activities towards both histidine and serine were determined with pyruvate as amino acceptor. Both activities were always found in the same fractions through all purification steps.

Preparation of crude extracts. Male rats (120–150g) of the Donryu strain were injected subcutaneously with glucagon suspended in 0.15% KCl (0.35mg, every 8h for 3 days) and killed by decapitation. The mitochondrial fractions (heavy and light mitochondria) were prepared from six livers (about 28g), and suspended in 150ml of 5mM-buffer. The suspension was sonicated for 4min at 20kHz with a Kubota Sonicator (Tokyo, Japan), and then centrifuged at 105000*g* for 30min.

DEAE-cellulose chromatography. The supernatant was applied to a column (5.0cm×6.0cm) of DEAE-cellulose, equilibrated with 5mM buffer. After washing with 800ml of 5mM buffer, the enzyme was eluted with 25mM buffer. The active fractions were pooled and concentrated by Diaflo ultrafiltration (Bioengineering Co., Tokyo, Japan) by using a G10T filter.

Isoelectric focusing. The concentrated enzyme solution was subjected to isoelectric focusing on a pH3.5–10 Ampholine gradient. The focusing resulted in the detection of a major peak with pI8.0 (isoenzyme 1) and a minor peak with pI5.2 (isoenzyme 2). Isoenzyme 1 fraction possessed aminotransferase activities towards both histidine and serine, whereas isoenzyme 2 fraction showed only histidine–pyruvate aminotransferase activity. The isoenzyme 1 fractions were pooled and concentrated by ultrafiltration.

Sephadex G-150 gel filtration. The concentrated enzyme solution was added to a Sephadex G-150 column (2.5cm×100cm), which had been equilibrated with 50mM buffer. The column was eluted with the same buffer at a flow rate of 25ml/h. The effluent was collected in 3.6ml fractions. The active fractions were pooled, concentrated by ultrafiltration and diluted with water to adjust the buffer concentration to 5mM.

Hydroxyapatite chromatography. The enzyme solution was applied to a hydroxyapatite column (2.5cm×1.5cm) which had been equilibrated with 5mM buffer. After washing with 50ml amounts of 15mM and then 50mM buffer, the enzyme was eluted with 50mM buffer. The active fractions were pooled and concentrated by ultrafiltration.

The results of the purification of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase are shown in Table 2. About 62-fold purification had been achieved, with a recovery of about 35% for both enzyme activities. Histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities of the purified enzyme were not increased by the addition of pyridoxal phosphate (0–40μM), showing the presence of the holoenzyme. The ratio of activity with histidine to that with serine remained constant during the purification. The purified enzyme (in 50mM-potassium phosphate buffer, pH7.5) may be stored at −20°C for at least 6 weeks without loss of either activity. Little or none of either activity was lost when the enzyme was stored at 0–5°C for 2 weeks. Heating the purified enzyme at 70°C for different lengths of time produced equivalent losses of both activities (Fig. 1).

On polyacrylamide-disc-gel electrophoresis at pH8.9 in 7% gel, the purified enzyme migrated toward the anode as a single protein band, which coincided with both histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities. Sephadex G-150 gel filtration (for molecular-weight determination) gave a single symmetrical peak, with protein and the two aminotransferase activities coincident. Similarly sedimentation in a sucrose density

Table 2. *Purification of histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase*

Details of purification and assay methods are given in the text.

		Histidine–pyruvate aminotransferase			Serine–pyruvate aminotransferase			Histidine/ serine activity ratio
	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	
Extract	375	12.6	4280	1	3.5	1240	1	3.8
DEAE-cellulose	29.0	130	3770	10.3	34.2	992	10.4	3.8
Isoelectric focusing and Sephadex G-150	3.1	622	1930	49.4	178	552	53.9	3.5
Hydroxyapatite	2.0	771	1540	61.2	208	416	63.0	3.7

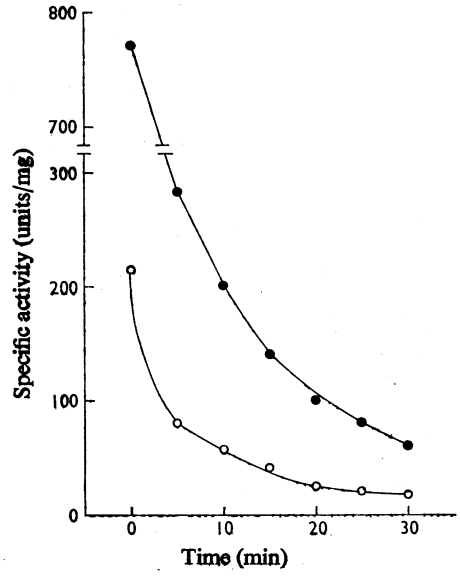

Fig. 1. *Effect of heat denaturation on the enzyme*

The purified enzyme was maintained at 70°C for different lengths of time before assays of histidine–pyruvate aminotransferase (●) and serine–pyruvate aminotransferase (○). Details of assays are given in the text.

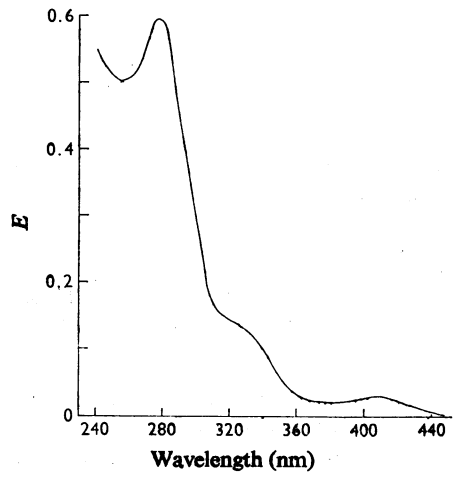

Fig. 2. *Absorption spectrum of the enzyme*

The protein concentration was 0.5 mg/ml. The buffer was 50 mM-potassium phosphate, pH 7.5.

gradient (for molecular-weight determination) showed a single peak for protein and the two aminotransferase activities.

The mol.wt. was estimated as 73000 ± 5000 by Sephadex G-150 gel filtration and as 75000 ± 6000 by sucrose-density-gradient centrifugation. This value was similar to that previously found for isoenzyme 1 from control rat liver mitochondria (Noguchi *et al.*, 1976*a*). Polyacrylamide-disc-gel electrophoresis in sodium dodecyl sulphate gave a single protein component that had an estimated mol.wt. of 38000 ± 3000, showing that isoenzyme 1 probably consists of two identical subunits.

The absorption spectrum of the purified enzyme showed three maxima (Fig. 2). That at 280 nm is typical of proteins and is caused by aromatic amino acid residues in the polypeptide chains. The peak at 410 nm is probably due to pyridoxal phosphate bound to the protein. We did not determine whether the shoulder at about 325 nm represents the pyridoxamine form (Taylor & Jenkins, 1966) or the pyridoxal form (Hayashi *et al.*, 1967). Dialysis (16 h at 4°C) of the purified enzyme against 20 mM-histidine or 20 mM-serine in 50 mM-potassium phosphate buffer, pH 7.5, did not lead to resolution of the cofactor from the enzyme. Further, when the enzyme was incubated at 37°C for 60 min in 0.5 M-potassium phosphate buffer, pH 7.0, in the presence of 20 mM-histidine, no

resolution was observed. These results show that the enzyme contains tightly bound pyridoxal phosphate.

Enzymic properties

pH-dependence. Over the pH range 8.0–9.6 histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities showed nearly identical profiles, with pH optimum at 9.0 (Fig. 3).

Substrate specificity. The data presented above strongly suggested that we were dealing with a single transaminase. It was therefore decided to examine its substrate specificity. The purified enzyme catalysed transamination between various amino acids and pyruvate or phenylpyruvate (Table 3). With 20mM-pyruvate as amino acceptor, the order of effectiveness of 20mM-L-amino acids was phenylalanine > histidine > tyrosine > serine. A little activity was observed with tryptophan and 5-hydroxytryptophan. With 1.4mM-phenylpyruvate, leucine, alanine, methionine, asparagine, glutamine, serine, ornithine and threonine were effective amino donors, but little or no activity was detected with glycine, isoleucine, valine, lysine, aspartate and glutamate. Transamination between aromatic L-amino acids and phenylpyruvate was not examined. Alanine–2-oxoglutarate aminotransferase and aspartate–2-oxoglutarate aminotransferase activities were not detected.

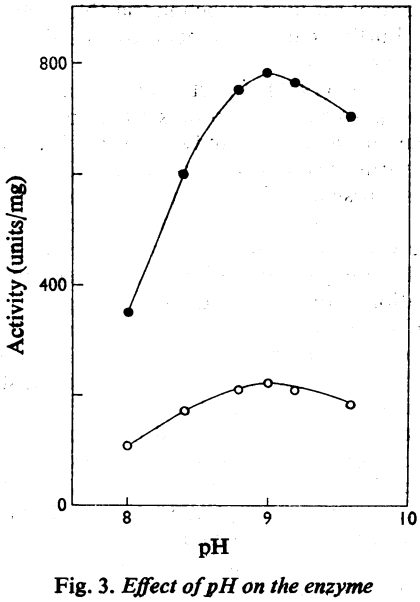

Fig. 3. *Effect of pH on the enzyme*

Assay conditions are as described in the text, except that Tris/HCl was used at pH 8.0–9.6. Histidine–pyruvate (●) and serine–pyruvate (○) aminotransferase activities are shown.

Table 3. *Specificity of the purified enzyme for various amino acids with pyruvate or phenylpyruvate*

Details of assay methods are given in the text.

Amino acid	Specific activity (units/mg) Pyruvate	Phenylpyruvate
Histidine	770	
Phenylalanine	1500	
Tyrosine	433	
Tryptophan	27.2	
5-Hydroxytryptophan	30.2	
Serine	213	43.7
Leucine		64.1
Isoleucine		0
Valine		2.2
Asparagine		62.3
Lysine		3.5
Glutamine		59.7
Methionine		62.9
Ornithine		42.7
Aspartic acid		3.9
Threonine		25.7
Alanine		63.3
Glycine		4.2
Glutamic acid		1.3

Table 4. *Specificity of the enzyme for various 2-oxo acids with histidine and serine*

Details of assays are given in the text.

2-Oxo acid	Specific activity (units/mg) Histidine	Serine
Pyruvate	765	213
Phenylpyruvate	—	192
Oxaloacetate	612	165

Serine and histidine were found to act as competitive inhibitors of histidine–pyruvate transamination and serine–pyruvate transamination respectively.

The amino-acceptor specificity of the enzyme was examined with histidine and serine (Table 4). The order of effectiveness of keto acid substrates (20mM) was pyruvate > phenylpyruvate > oxaloacetate with serine, and pyruvate > oxaloacetate with histidine. Histidine–phenylpyruvate aminotransferase activity was not examined because of the difficulty of its assay. 2-Oxoglutarate was inactive with both histidine and serine.

Inhibitors. Histidine–pyruvate and serine–pyruvate transamination activities, each to the same degree, were inhibited by carbonyl reagents, namely isonicotinic acid hydrazide, hydroxylamine, KCN and semicarbazide (Table 5); this inhibition is probably

Table 5. *Effects of various reagents on the enzyme*

Details of assays are given in the text.

Reagent added	Concn. (mM)	Relative activity: Histidine–pyruvate aminotransferase	Relative activity: Serine–pyruvate aminotransferase
Control		100	100
Isonicotinic acid hydrazide	0.5	47.2	43.9
	1.0	30.7	28.1
Hydroxylamine	2.0	6.0	4.3
	10.0	0	0
Semicarbazide	1.0	40.0	38.9
	5.0	13.9	14.0
KCN	1.0	52.1	48.9
	5.0	0.8	0.4

due to the binding of the inhibitor with the aldehyde group of the coenzyme, pyridoxal phosphate.

Discussion

Histidine–pyruvate aminotransferase (Spolter & Baldridge, 1963, 1964; Schirmer & Harper, 1970; Lee & Harper, 1971; Morris *et al.*, 1973), phenylalanine–pyruvate aminotransferase (Civen *et al.*, 1967; Brown & Civen, 1969; Fuller *et al.*, 1972*a*,*b*, 1973, 1974) and serine–pyruvate aminotransferase (Rowsell *et al.*, 1969, 1972, 1973; Cheung *et al.*, 1969; Sallach *et al.*, 1972) of rat liver have been independently studied. All three enzymes are induced by the injection of glucagon. We have previously reported that the mitochondrial and supernatant fractions of rat liver contain two forms of histidine–pyruvate aminotransferase (isoenzymes 1 and 2) and that isoenzyme 1 is identical with glucagon-inducible phenylalanine–pyruvate aminotransferase (Okuno *et al.*, 1975; Noguchi *et al.*, 1976*a*). Subsequently, Shih *et al.* (1976) reported that rat liver supernatant histidine–pyruvate aminotransferase was identical with the supernatant phenylalanine–pyruvate aminotransferase.

In the present study, with liver mitochondria of glucagon-injected rats as starting material, histidine–pyruvate aminotransferase (isoenzyme 1) and serine–pyruvate aminotransferase were co-purified to homogeneity as judged by polyacrylamide-gel electrophoresis in the absence and in the presence of sodium dodecyl sulphate, gel filtration, sucrose-density-gradient centrifugation and isoelectric focusing. Transamination activity with histidine maintained a constant ratio to that with serine during purification, which was unchanged by a variety of treatments of the purified enzyme. Histidine–pyruvate aminotransferase and serine–pyruvate aminotransferase activities were competitively inhibited by serine and histidine respectively. These data show that isoenzyme 1 is identical with serine–pyruvate aminotransferase.

We have reported that the mitochondrial isoenzyme 1 preparations from livers of control and glucagon-injected rats are remarkably similar with respect to pH profile, electrophoretic migration, molecular weight and substrate specificity, suggesting the identity of the two enzymes. However, the specific activity (histidine–pyruvate transamination) of the isoenzyme 1 preparation purified to homogeneity from glucagon-treated rat liver mitochondria in the present study was much higher than that previously found for the highly purified isoenzyme 1 preparation from control rat liver mitochondria (Noguchi *et al.*, 1976*a*). Further detailed comparative studies of both enzymes are needed.

Shih *et al.* (1976) report that partially purified histidine–pyruvate aminotransferase of rat liver supernatant fraction showed aminotransferase activities towards histidine and phenylalanine, but not towards tyrosine, serine, methionine, glutamine and alanine with pyruvate as amino acceptor. Their enzyme would appear to be isoenzyme 1 because it was purified from glucagon-injected rat liver, but their observations on amino-donor specificity differ markedly from those recorded in the present work. Our isoenzyme 1 preparation showed high aminotransferase activities with pyruvate towards histidine, phenylalanine, tyrosine and serine. The transamination activity with pyruvate and other amino acids, except tryptophan and 5-hydroxytryptophan, was not examined in the present investigation. With phenylpyruvate as amino acceptor, isoenzyme 1 catalysed transamination with leucine, alanine, methionine, asparagine, glutamine, serine, ornithine and threonine. This high activity towards glutamine might suggest the identity of isoenzyme 1 with glutamine aminotransferase (EC 2.6.1.15), which uses pyruvate and phenylpyruvate as effective amino acceptors. This enzyme has been highly purified from the kidney

(Cooper & Meister, 1974), liver (Cooper & Meister, 1972) and brain (Vanleuven, 1975) of rats. However, isoenzyme 1 has a lower molecular weight than that reported for glutamine aminotransferase from the brain, kidney or liver; and isoenzyme 1 is present only in the liver of rats, not in the brain, kidney and heart (Noguchi *et al.*, 1976*b*). Moreover, we have found that glutamine aminotransferase of the liver, kidney, brain and heart of rats has an isoelectric point (pI 5.2) identical with that of isoenzyme 2 (not detailed in the Results section). These data show that isoenzyme 1 is different from glutamine aminotransferase and they raise the possibility that isoenzyme 2 may be identical with glutamine aminotransferase.

Sallach *et al.* (1972) reported that rat liver serine–pyruvate aminotransferase activity was elevated in the gluconeogenic state brought about by administration of cortisone, glucagon or cyclic AMP and by alloxan-diabetes. Rowsell *et al.* (1973) described that rat liver serine–pyruvate aminotransferase activity markedly exceeded the normal adult value in the neonatal period, after glucagon injection and alloxan injection, suggesting a role of the enzyme in gluconeogenesis. The association of isoenzyme 1 with serine–pyruvate aminotransferase suggests the need to investigate the role of isoenzyme 1 in gluconeogenesis.

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