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Purification, Characterization and Identification of Rat Liver Histidine–Pyruvate Aminotransferase Isoenzymes

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1. Histidine–pyruvate aminotransferase (isoenzyme 1) was purified to homogeneity from the mitochondrial and supernatant fractions of rat liver, as judged by polyacrylamide-gel electrophoresis and isoelectric focusing. Both enzyme preparations were remarkably similar in physical and enzymic properties. Isoenzyme 1 had pI 8.0 and a pH optimum of 9.0. The enzyme was active with pyruvate as amino acceptor but not with 2-oxoglutarate, and utilized various aromatic amino acids as amino donors in the following order of activity: phenylalanine > tyrosine > histidine. Very little activity was found with tryptophan and 5-hydroxytryptophan. The apparent K_m values were about 2.6mM for histidine and 2.7mM for phenylalanine. K_m values for pyruvate were about 5.2mM with phenylalanine as amino donor and 1.1mM with histidine. The aminotransferase activity of the enzyme towards phenylalanine was inhibited by the addition of histidine. The mol.wt. determined by gel filtration and sucrose-density-gradient centrifugation was approx. 70000. The mitochondrial and supernatant isoenzyme 1 activities increased approximately 25-fold and 3.2-fold respectively in rats repeatedly injected with glucagon for 2 days. 2. An additional histidine–pyruvate aminotransferase (isoenzyme 2) was partially purified from both the mitochondrial and supernatant fractions of rat liver. Nearly identical properties were observed with both preparations. Isoenzyme 2 had pI 5.2 and a pH optimum of 9.3. The enzyme was specific for pyruvate and did not function with 2-oxoglutarate. The order of effectiveness of amino donors was tyrosine = phenylalanine > histidine > tryptophan > 5-hydroxytryptophan. The apparent K_m values for histidine and phenylalanine were about 0.51 and 1.8mM respectively. K_m values for pyruvate were about 3.5mM with phenylalanine and 4.7mM with histidine as amino donors. Histidine inhibited phenylalanine aminotransferase activity of the enzyme. Gel filtration and sucrose-density-gradient centrifugation yielded a mol.wt. of approx. 90000. Neither the mitochondrial nor the supernatant isoenzyme 2 activity was elevated by glucagon injection.

It has been reported that histidine–pyruvate aminotransferase (EC 2.6.1.–) is present in both the mitochondrial and supernatant fractions of rat liver, and both activities are elevated by the injection of glucagon or cyclic AMP (Spolter & Baldridge, 1964; Morris *et al.*, 1973). The two enzymes differ in K_m values for histidine and pyruvate, in pH profile and in heat lability.

Phenylalanine–pyruvate aminotransferase (EC 2.6.1.–) activity is also found in rat liver, and elevated by glucagon or cyclic AMP (Civen *et al.*, 1967; Brown & Civen, 1969; Fuller *et al.*, 1972, 1974). Morris *et al.* (1973) reported that compared with livers from control rats, livers from glucagon-injected rats exhibited a virtually identical increase in phenylalanine–pyruvate and histidine–pyruvate aminotransferase activities both in the mitochondria and in the cytosol. However, there are few reports on the purification and properties of histidine–pyruvate aminotransferase and phenylalanine–pyruvate aminotransferase. The present report describes the purification, properties and identity of the two enzymes from rat liver.

Experimental

Materials

The following compounds were obtained from commercial sources: L-phenylalanine, L-tyrosine, L-histidine and L-tryptophan, from Tanabe Amino Acid Foundation, Osaka, Japan; sodium pyruvate and pyridoxal 5′-phosphate, from Nakarai Chemicals, Kyoto, Japan; monosodium 2-oxoglutarate and crystalline glucagon, from Sigma Chemical Co., St. Louis, MO, U.S.A.; 5-hydroxy-L-tryptophan, from Calbiochem, San Diego, CA, U.S.A.; DEAE-cellu-

lose and hydroxyapatite, from Seikagaku Kogyo Co., Tokyo, Japan; Sephadex G-150, from Pharmacia Fine Chemicals, Uppsala, Sweden; Ampholine carrier ampholytes for electrofocusing, from LKB Produkter AB, Stockholm, Sweden.

Methods

Enzyme assays. Aminotransferase assays towards histidine, phenylalanine, tyrosine, tryptophan and 5-hydroxytryptophan were based on the arsenate-catalysed formation of aromatic 2-oxo acid–enol–borate complexes, which show characteristic absorption spectra in the 300nm region (Lin *et al.*, 1958). Assay procedures were as described by George *et al.* (1967). The assay mixtures (0.8ml) contained, unless specified otherwise, 20mM-L-amino acid, 20mM-pyruvate, 40μM-pyridoxal 5'-phosphate, enzyme preparation and 0.2M-Tris/HCl, pH9.0. In the blank, pyruvate was added after incubation. After incubation at 37°C, 0.2ml of 20% (w/v) trichloroacetic acid was added to the reaction mixture. After centrifugation, a 0.5ml portion of the supernatant was mixed with 2.5ml of the arsenate–borate reagent (George *et al.*, 1967) and thoroughly mixed. After the mixture had been left for 30min at room temperature (20°C), the extinction increase was measured at 292nm with histidine, 300nm with phenylalanine, 310nm with tyrosine, 330nm with tryptophan and 334nm with 5-hydroxytryptophan as amino donor. The amount of aromatic 2-oxo acid formed was calculated from the molar extinction coefficient of the relevant enol–borate complex [11300 for imidazolepyruvate, 7100 for phenylpyruvate, 10700 for *p*-hydroxyphenylpyruvate, 10800 for indolepyruvate, 9700 for 5-hydroxyindolepyruvate (Jacoby & La Du, 1964)]. Enzyme activity was calculated from the linear part of the progress curve. A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1μmol of aromatic 2-oxo acid/h under the above conditions. Specific activity is defined as enzyme units per mg of protein. The apparent K_m values were determined from double-reciprocal plots of initial velocity and substrate concentration.

Phenylalanine–pyruvate aminotransferase was also assayed in the presence of histidine. After incubation for 1h at 37°C, 0.2ml of 20% (w/v) trichloroacetic acid was added to the reaction mixture (0.8ml), which was then applied to a column (0.5cm×1cm) of Dowex 50 (H^+; 8% cross-linkage; 200–400 mesh) and washed through with 1.5ml of water. By this method, 95–98% of the phenylpyruvate was recovered in the flow-through and washings, whereas imidazolepyruvate was completely retained on the Dowex 50 resin. The flow-through and washings were combined, and a 1ml portion was mixed with 2.0ml of the arsenate–borate reagent (George *et al.*, 1967). The amount of phenylpyruvate–borate complex formed was then measured.

Catalase (H_2O_2–H_2O_2 oxidoreductase, EC1.11.1.6) (Chance & Maehly, 1955), alcohol dehydrogenase (alcohol–NAD^+ oxidoreductase, EC 1.1.1.1) (Büttner, 1965) and monoamine oxidase [monoamine–oxygen oxidoreductase (deaminating), EC 1.4.3.4] (Tottmar *et al.*, 1973) were assayed as described in the cited references.

Subcellular fractionation. Male rats (60–80g) of the Donryu strain were used. Subcellular fractions of rat liver were obtained by the method of de Duve *et al.* (1955).

Protein determination. The protein content 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.

Polyacrylamide-disc-gel electrophoresis. Electrophoresis was performed in a continuous 7% total polyacrylamide-gel column (0.5cm×8cm) containing 0.184% bisacrylamide, by the original procedures of Davis (1964). A Tris/glycine buffer, pH8.9 (11.0g of Tris and 14.4g of glycine/litre) was used as the running buffer, and Bromophenol Blue was used as tracking dye. Electrophoresis was carried out at 4°C for 180min at a current of 2.0mA/column. The sample was applied in 20% (w/v) sucrose solution. For enzyme assay immediately after electrophoresis the gels were cut into 2mm slices and these were added individually to the assay medium.

Isoelectric focusing. Isoelectric focusing was in principle carried out by the method of Vesterberg & Svensson (1966). Runs were carried out in a 100ml column on a pH3.5–10 Ampholine gradient (2%, w/v). The enzyme solution was added to the dense and less dense sucrose solutions before gradient formation. The potential was maintained at 500V for 48h at 4°C. Fractions (2.0ml) were collected; enzyme activity and pH determinations were performed immediately.

Approximate molecular-weight determination by sucrose-density-gradient centrifugation. The method of Martin & Ames (1961) was used with sucrose gradients (5–20%, w/v) in 0.1M-potassium phosphate buffer, pH7.5. Centrifugation was carried out at 39000rev./min (135000*g*) with a RP 50 swinging-bucket rotor in a Hitachi 65P centrifuge (Hitachi Ltd., Tokyo, Japan) at 4°C for 15h. Fractions (three drops) were collected by upward displacement of the tube contents (4.8ml) by using a Mitsumi Gradienter SJ-1300 UD (Mitsumi Scientific Industry Co., Tokyo, Japan), and analysed for protein and enzyme activities. Bovine catalase was used as internal standard with an assumed $s_{20,w} = 11.3$S.

Analytical gel filtration. Gel filtration on a Sephadex G-150 column (2.5cm×100cm) was calibrated with the following proteins of known mol.wt.: cytochrome *c*, 13000; ovalbumin, 45000; bovine serum albumin, 67000; catalase, 235000. The various proteins were

detected either by extinction measurement or by enzyme activity. Elution volumes were plotted against the logarithm of the respective molecular weights (Andrews, 1964).

Results

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

Table 1 shows the distribution of histidine–pyruvate aminotransferase and phenylalanine–pyruvate aminotransferase activities in the subcellular fractions of rat liver. Both enzyme activities were similarly distributed and mainly found in the mitochondrial and supernatant fractions.

Subcellular distribution of marker enzymes

Monoamine oxidase was used as the mitochondrial marker and alcohol dehydrogenase as the cytosol marker. Recoveries of monoamine oxidase and alcohol dehydrogenase were 101.9 and 99.4% respectively. The percentage distribution of monoamine oxidase was as follows: nuclear fraction, 14.1%; heavy-mitochondrial fraction, 62.0%; light-mitochondrial fraction, 17.4%; microsomal fraction, 7.1%; supernatant fraction, 1.3%. Most (95.2%) of the alcohol dehydrogenase was in the supernatant fraction. No activity was detected in the mitochondrial fraction and very little activity in other particulate fractions. The distribution of the two marker enzymes corresponded well to the results obtained by Tottmar *et al.* (1973).

Purification of histidine–pyruvate aminotransferase isoenzymes

All manipulations were carried out at 0–4°C. Glass-distilled water and potassium phosphate buffer, pH7.5, containing 100μM-pyridoxal 5′-phosphate, were used throughout. Histidine–pyruvate aminotransferase and phenylalanine–pyruvate aminotransferase activities were always recovered in the same fraction throughout the purifications, from both the mitochondrial and supernatant initial preparations.

Purification of the mitochondrial isoenzyme 1 *and isoenzyme* 2. Rats were stunned, decapitated and bled. The mitochondrial fractions (heavy-mitochondrial and light-mitochondrial fractions) were prepared from 95 livers, and suspended in 250ml 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.

The supernatant was applied to a column (5.0cm × 20cm) of DEAE-cellulose, equilibrated with 5mM buffer. After washing with 500ml of 5mM and 15mM buffer, histidine–pyruvate aminotransferase activity was eluted with 50mM buffer. The active fractions were pooled and concentrated by Diaflo ultrafiltration (Bio-engineering Co, Tokyo, Japan) by using a G 10 T filter, and diluted with water to a buffer concentration of 5mM.

The enzyme solution was applied to a DEAE-cellulose column (5.0cm × 8cm), equilibrated with 5mM buffer. After washing with 500ml of 5mM buffer, the enzyme was eluted with a 500ml linear gradient of 5–50mM buffer. Fractions (5ml) were collected at a flow rate of 70ml/h. The active fractions were pooled and concentrated by ultrafiltration.

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.

The concentrated enzyme solution was subjected to isoelectric focusing on a pH3.5–10 Ampholine gradient as described under 'Methods'. The focusing resulted in the detection of two activity peaks with isoelectric points of pH8.0 and 5.2 respectively,

Table 1. *Intracellular distribution of histidine–pyruvate aminotransferase and phenylalanine–pyruvate aminotransferase*

Experimental details are as described in the Experimental section.

Fraction	Protein (mg/g of wet tissue)	Histidine–pyruvate aminotransferase		Phenylalanine–pyruvate aminotransferase	
		Specific activity (units/mg)	Total activity (%)	Specific activity (units/mg)	Total activity (%)
Homogenate	145.0	0.11	100	0.29	100
Nuclei	22.8	0.10	14.3	0.19	10.3
Heavy mitochondria	28.7	0.18	32.4	0.36	24.6
Light mitochondria	15.4	0.35	33.8	0.94	34.4
Microsomal fraction	24.8	0.01	1.6	0.07	4.1
Supernatant	62.3	0.06	23.4	0.15	22.2
Recovery	154.0		105.5		95.6

possessing aminotransferase activities towards both histidine and phenylalanine (Fig. 1). The enzyme with pI8.0 is hereafter designated isoenzyme 1 and that with pI5.2 isoenzyme 2. The active fractions under each peak were pooled, applied to separate columns of Sephadex G-150 and eluted as described above. The active fractions of isoenzyme 1 and isoenzyme 2 were pooled separately, and concentrated to about 0.5ml by ultrafiltration.

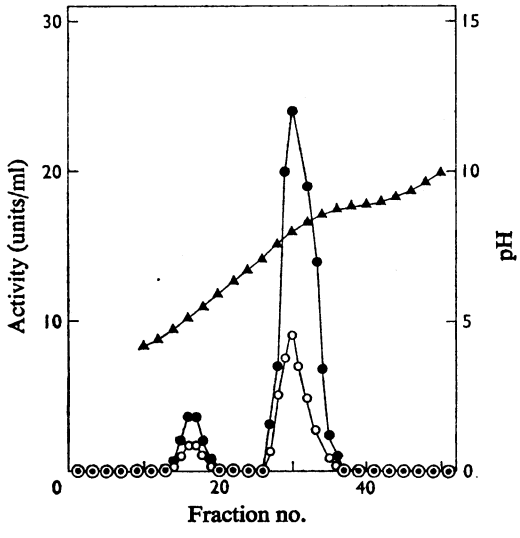

Fig. 1 *Isoelectric focusing of the mitochondrial enzyme preparation obtained after Sephadex G-150 gel filtration*

The mitochondrial enzyme preparation from a Sephadex G-150 column was subjected to isoelectric focusing on a pH3.5–10 Ampholine gradient as described in the text. Fractions (2ml) were collected. pH values (▲) and aminotransferase activities towards histidine (○) and phenylalanine (●) were determined as described in the text.

The concentrated isoenzyme 1 solution was made up to a volume of 0.6ml with water, and fractions (0.1ml) were layered on each of six 4.5ml linear sucrose gradients [5–20% (w/v) sucrose in 0.1M-potassium phosphate buffer, pH7.5]. After centrifugation as described under 'Methods', the solution was fractionated and assayed for enzyme activities. A single peak was obtained, which possessed both histidine–pyruvate and · phenylalanine–pyruvate aminotransferase activities. The active fractions were pooled, dialysed overnight against 5 litres of 5mM buffer and concentrated by ultrafiltration.

The overall purifications of isoenzymes 1 and 2 are summarized in Table 2. Isoenzyme 1 was purified about 760-fold with histidine as amino donor and 860-fold with phenylalanine, compared with the homogenate. The purification of isoenzyme 2 was about 27-fold for histidine as the amino donor and 23-fold for phenylalanine.

Purification of the supernatant isoenzymes 1 and 2. Livers from 45 rats were homogenized with 450ml of 0.25M-sucrose in 5mM-potassium phosphate buffer, pH7.5, and then centrifuged at 105000*g* for 60min. Subsequently, the supernatant isoenzymes 1 and 2 were purified by the same procedures as described for the mitochondrial isoenzymes. The isoelectric-focusing profile of the enzyme preparation from a Sephadex G-150 column was virtually identical with that obtained for the mitochondrial isoenzymes. Sucrose-density-gradient centrifugation of isoenzyme 1 after isoelectric-focusing step resulted in the detection of a

Table 2. *Purification of the mitochondrial isoenzymes 1 and 2*

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

		Histidine–pyruvate aminotransferase			Phenylalanine–pyruvate aminotransferase		
	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Specific activity (units/mg)	Total activity (units)	Purification (fold)
Homogenate	44590	0.11	4905	1	0.27	12039	1
Crude extract	2730	0.35	956	3.2	0.95	2594	3.5
First DEAE-cellulose	350	2.4	840	22	6.7	2345	24
Second DEAE-cellulose	92	5.0	460	45	14	1288	52
Sephadex G-150	29	12	348	109	33	957	122
Isoelectric focusing and Sephadex G-150							
Isoenzyme 1	1.1	75	83	682	208	229	770
Isoenzyme 2	4.5	3.0	14	27	6.3	28	23
Sucrose-density-gradient centrifugation							
Isoenzyme 1	0.8	83	66	755	231	185	856

Table 3. *Purification of the supernatant isoenzymes* 1 *and* 2

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

	Total protein (mg)	Histidine–pyruvate aminotransferase			Phenylalanine–pyruvate aminotransferase		
		Specific activity (units/mg)	Total activity (units)	Purification (fold)	Specific activity (units/mg)	Total activity (units)	Purification (fold)
Supernatant	9469	0.08	758	1	0.18	1704	1
First DEAE-cellulose	1125	0.45	506	5.6	1.0	1125	5.6
Second DEAE-cellulose	215	1.6	344	20	3.9	839	22
Sephadex G-150	41	5.3	217	66	12.2	500	68
Isoelectric focusing and Sephadex G-150							
Isoenzyme 1	1.4	44	77	688	144	202	800
Isoenzyme 2	11	2.0	22	25	4.1	45	23
Sucrose-density-gradient centrifugation							
Isoenzyme 1	0.6	76	46	950	198	119	1100

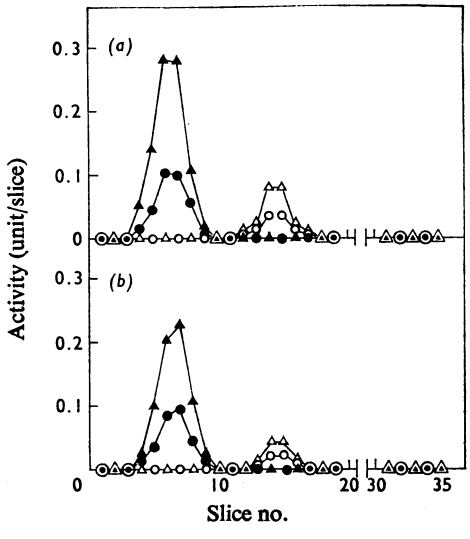

Fig. 2. *Polyacrylamide-gel electrophoresis of isoenzymes* 1 *and* 2

Electrophoresis, as described in the text, was carried out with the purified isoenzymes obtained after isoelectric focusing and Sephadex G-150 filtration (see Tables 2 and 3). A mixture of isoenzyme 1 (5μg) and isoenzyme 2 (50μg) was used with each gel. Electrophoretic profiles are shown of (*a*) the mitochondrial isoenzymes and (*b*) the supernatant isoenzymes. Aminotransferase activities of isoenzyme 1 towards histidine (●) and phenylalanine (▲), and those of isoenzyme 2 towards histidine (○) and phenylalanine (△) were determined after electrophoresis as described in the text.

single peak, which possessed aminotransferase activities towards both phenylalanine and histidine.

The overall purification procedures for isoenzymes 1 and 2 are summarized in Table 3. About 950-fold purification was obtained for isoenzyme 1 with histidine as amino donor and a 25-fold purification for isoenzyme 2. The purification of phenylalanine aminotransferase activity was about 1100-fold for isoenzyme 1 and 23-fold for isoenzyme 2.

Physical properties

Criteria of purity. The mitochondrial and supernatant isoenzyme 1 preparations were nearly identical in specific activity, in electrophoretic migration and in isoelectric-focusing profile. On polyacrylamide-disc-gel electrophoresis at pH8.9 in 7% gel, isoenzyme 1 migrated to the anode as a single protein band, which coincided with both histidine–pyruvate and phenylalanine–pyruvate aminotransferase activities after the gels were sliced (Fig. 2). Isoelectric focusing of the enzyme gave a single symmetrical peak (pI8.0), with protein and both enzyme activities coincident.

Identical electrophoretic migrations and isoelectric points (pH5.2) were observed for the mitochondrial and supernatant isoenzyme 2 preparations. Polyacrylamide-disc-gel electrophoresis (pH8.9, 7% gel) gave multiple protein-staining components, one of which coincided with activities toward both histidine and phenylalanine. The enzyme migrated faster than isoenzyme 1 to the anode (Fig. 2). Isoelectric focusing of the enzyme gave a single symmetrical peak (pI5.2) with both enzyme activities. The ratio of aminotransferase activity toward histidine and phenylalanine was constant for both purified isoenzyme 1 and 2 after electrophoresis and isoelectric focusing.

Molecular weight. The mol.wt. of the mitochondrial and supernatant isoenzyme 1 was estimated as approx. 70000 by Sephadex G-150 gel filtration, and for isoenzyme 2 as approx. 90000. Sucrose-density-

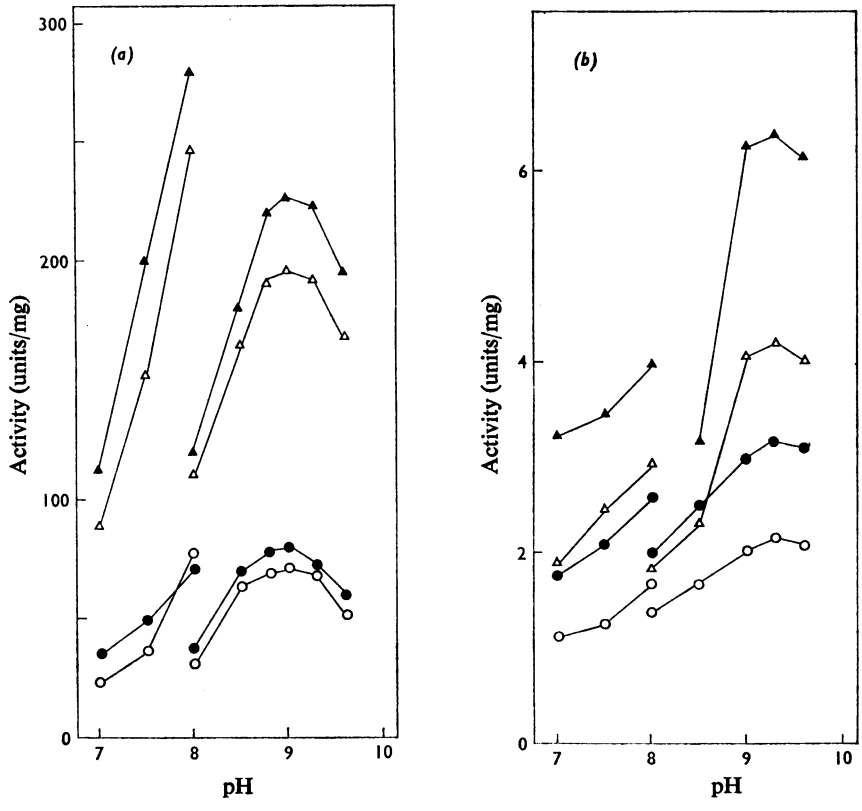

Fig. 3. *Effect of pH on isoenzymes* 1 *and* 2

(*a*) pH–activity curves of isoenzyme 1; (*b*) those of isoenzyme 2. Assay conditions were as described in the text, except that potassium phosphate buffer was used at pH 7.0–8.0 and Tris/HCl at pH 8.0–9.6. Histidine–pyruvate (●) and phenylalanine–pyruvate (▲) aminotransferase activities of the mitochondrial isoenzymes, and histidine–pyruvate (○) and phenylalanine–pyruvate (△) aminotransferase activities of the supernatant isoenzymes are shown.

Table 4. *Relative activities of the mitochondrial and supernatant isoenzymes* 1 *and* 2 *for various aromatic amino acids*

Assay conditions were as described in the text except that the final concentration of each L-amino acid was 6.5 mM. Relative activity values are given, signifying transamination rates compared with that for L-histidine.

	Isoenzyme 1		Isoenzyme 2	
L-Amino acid	Mitochondria	Supernatant	Mitochondria	Supernatant
Tyrosine	1.2	1.4	1.5	1.6
Phenylalanine	2.4	2.5	1.6	1.7
Histidine	1	1	1	1
Tryptophan	0.05	0.07	0.42	0.35
5-Hydroxytryptophan	0.13	0.15	0.20	0.15

gradient centrifugation gave a mol.wt. of approx. 67000 for isoenzyme 1 and approx. 88000 for isoenzyme 2, from both subcellular fractions.

Enzymic properties

The mitochondrial and supernatant isoenzyme 1 preparations were nearly identical with respect to pH optimum, K_m value for histidine, phenylalanine and pyruvate, degree of inhibition by a common substrate and relative activities with different amino donors. Marked differences were observed with isoenzyme 2. However, isoenzyme 2 preparations from both subcellular fractions were nearly identical in these properties.

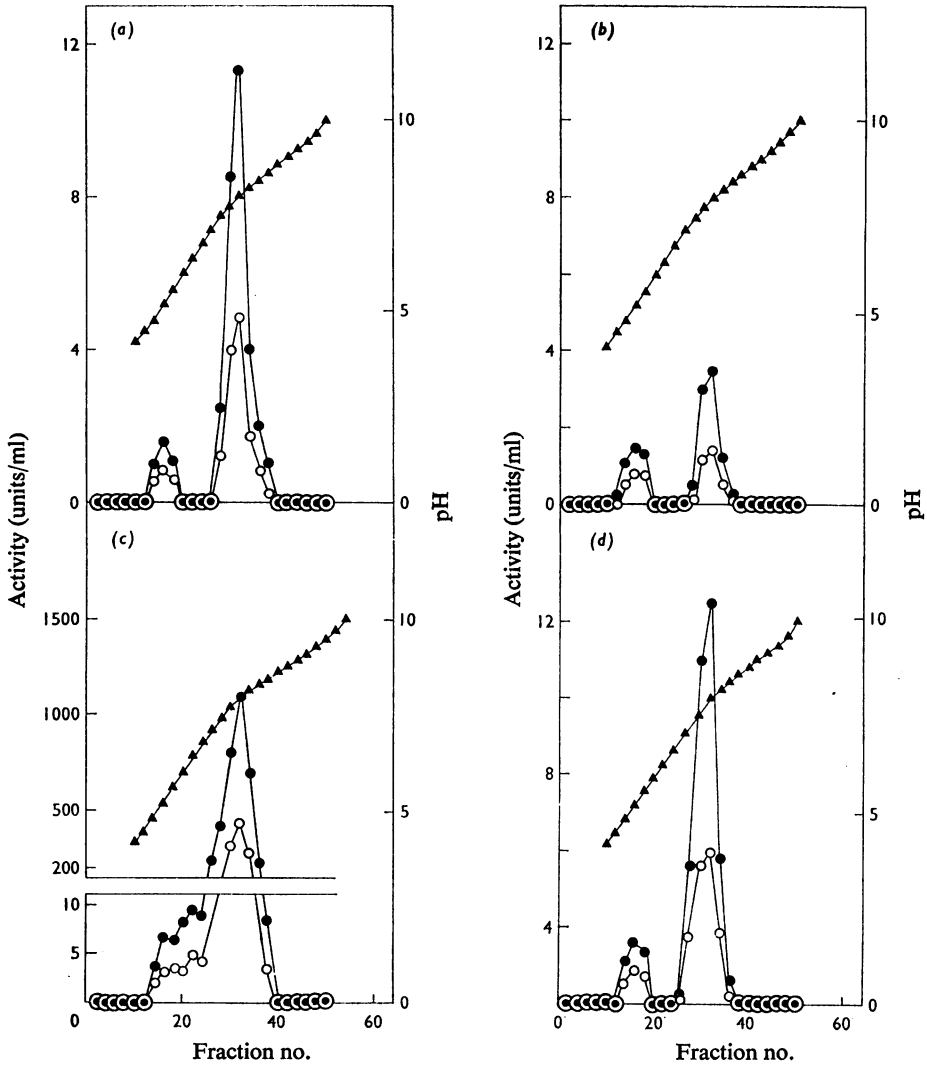

Fig. 4. *Response of the mitochondrial and supernatant isoenzymes to glucagon*

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 (0.35mg, 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, chilled, washed and homogenized in 3vol. of ice-cold 0.25M-sucrose in a Potter–Elvehjem tissue grinder with a Teflon pestle. The mitochondrial pellet (heavy-mitochondrial and light-mitochondrial pellets) and the cytosol fraction were prepared by the method of de Duve *et al.* (1955). The mitochondrial pellet was homogenized with 5mM-potassium phosphate buffer, pH7.5, equal in volume to the original sucrose homogenate and sonicated at 20kHz for 4min. The homogenate was centrifuged at 105000*g* for 30min, and the sediment discarded. The resulting mitochondrial extract and the cytosol fraction were separately concentrated by ultrafiltration. The concentrated mitochondrial extract was subjected to isoelectric focusing on a pH3.5–10 Ampholine gradient in amounts corresponding to one liver, and the concentrated cytosol fraction in amounts corresponding to half a liver. Fractions (2ml) were collected. pH values (▲) and aminotransferase activities towards both histidine (○) and phenylalanine (●) were determined as described in the text. Control, mitochondria (*a*); control, supernatant (*b*); +glucagon, mitochondria (*c*); +glucagon, supernatant (*d*).

pH optimum. Aminotransferase activities towards both histidine and phenylalanine were investigated as a function of pH over the range pH 7.0–9.6. Potassium phosphate buffer was used between pH 7.0 and 8.0, and Tris/acetate between pH 8.0 and 9.6. Histidine–pyruvate and phenylalanine–pyruvate aminotransferase activities showed nearly identical pH profiles for both isoenzymes (Fig. 3). The change from phosphate buffer to Tris/acetate at pH 8.0 resulted in a decrease of activity towards both phenylalanine and histidine with both isoenzymes. The pH optimum was 9.0 for isoenzyme 1 and 9.3 for isoenzyme 2.

Substrate specificity. With histidine and phenylalanine as amino donors, isoenzymes 1 and 2 were both specific for pyruvate as amino acceptor, exhibiting no activity with 2-oxoglutarate. The relative initial velocities with various amino acids (6.5mM) were determined by using 20mM-pyruvate as amino acceptor (Table 4). The order of effectiveness of these amino acids was phenylalanine > tyrosine > histidine > 5-hydroxytryptophan > tryptophan for isoenzyme 1, and tyrosine = phenylalanine > histidine > tryptophan > 5-hydroxytryptophan for isoenzyme 2. The isoenzymes differed in apparent K_m values. K_m values of the mitochondrial (supernatant) isoenzyme 1 were 2.7mM (2.6mM) for phenylalanine, 2.6mM (2.6mM) for histidine, 5.2mM (5.5mM) for pyruvate with phenylalanine as amino donor, and 1.1mM (0.9mM) for pyruvate with histidine. Corresponding values of the mitochondrial (supernatant) isoenzyme 2 were 1.8mM (2.0mM), 0.6mM (0.6mM), 3.5mM (3.7mM) and 4.7mM (4.5mM) respectively. With 6.5mM-phenylalanine and 20mM-pyruvate, phenylalanine aminotransferase activities of isoenzymes 1 and 2 were inhibited about 44 and 40% respectively, by the addition of 5mM-histidine.

Response of the mitochondrial and supernatant isoenzymes to the injection of rats with glucagon

The response of the mitochondrial and supernatant isoenzymes to glucagon was examined by using an isoelectric-focusing technique. Fig. 4 shows the focusing profiles of the mitochondrial extracts and the supernatant fractions from livers of both control and glucagon-injected rats. In each case, histidine–pyruvate and phenylalanine–pyruvate aminotransferase activities were identically focused. The two separated activity peaks of isoenzymes 1 and 2 were obtained with both the mitochondrial extract and the supernatant fraction from control liver, and with the supernatant fraction from glucagon-treated rat liver. However, there was evidence of an additional activity peak with pI 6.2 between isoenzymes 1 and 2, with the mitochondrial extract from glucagon-treated rat liver. This isoenzyme was not further characterized in the present study.

In comparison with isoenzyme 1 of control liver, isoenzyme 1 of glucagon-treated rat liver exhibited virtually identical percentage increases in total histidine–pyruvate and phenylalanine–pyruvate aminotransferase activity in both the mitochondrial and supernatant fractions. The mitochondrial and supernatant isoenzyme 1 activities were elevated about 25-fold and 3.2-fold respectively. However, isoenzyme 2 activity was not affected by glucagon injection in either subcellular fraction.

Properties of isoenzyme 1 from liver of glucagon-injected rats

Isoenzyme 1 fractions 28–36 obtained after the electrofocusing of the mitochondrial extract or the supernatant fraction from glucagon-treated liver (Fig. 4) were pooled separately and applied to separate columns (2.5cm × 100cm) of Sephadex G-150 for removal of Ampholine. The properties of each resulting enzyme solution were compared with those of isoenzyme 1 preparations (Tables 2 and 3) purified from control liver. Both the mitochondrial and supernatant isoenzyme 1 from glucagon-treated liver were remarkably similar to those from control liver in isoelectric point, molecular weight, pH optimum, electrophoretic migration, activity ratio toward histidine, phenylalanine, tyrosine, tryptophan and 5-hydroxytryptophan, K_m values for histidine, phenylalanine and pyruvate, and degree of inhibition of phenylalanine aminotransferase activity by histidine. These results suggest that isoenzyme 1 from glucagon-treated rat liver is identical with that from control rat liver.

Discussion

The presence of two distinct isoenzymes of histidine–pyruvate aminotransferase in rat liver has been documented. One is localized in the mitochondria; the other is restricted to the cytosol. Spolter & Baldridge (1964) reported that the two isoenzymes differed in K_m value for histidine and pyruvate, in pH profile and in heat lability. Morris *et al.* (1973) suggested that the mitochondrial and cytosol histidine–pyruvate aminotransferases are different proteins on the basis of the different response of the two activities to hormones, and noting the findings of Spolter & Baldridge (1964). However, all these observations were made on crude enzyme systems. In the present study, physical and enzymic properties of rat liver histidine–pyruvate aminotransferases were investigated by using purified enzyme preparations. The mitochondrial and supernatant fractions were each found to contain two forms of histidine–pyruvate aminotransferase with pI 8.0 for isoenzyme 1 and 5.2 for isoenzyme 2 respectively. The mitochondrial isoenzyme 1 was nearly identical with the supernatant isoenzyme 1, and the mitochondrial isoenzyme 2 with the supernatant isoenzyme 2, with respect to pH profile, isoelectric point, electrophoretic migra-

tion, molecular weight and substrate specificity. Marked differences were observed between isoenzymes 1 and 2.

It has been reported that rat liver histidine–pyruvate and phenylalanine–pyruvate aminotransferase activities are increased by glucagon injection, and these increases may be mediated by intracellular cyclic AMP (Fuller *et al.*, 1972, 1974; Morris *et al.*, 1973). In the present study, both the mitochondrial and cytosol isoenzyme 1 were found to be increased by glucagon injection, but not isoenzyme 2. Isoenzyme 1 from liver of glucagon-injected rats had a very similar activity ratio toward histidine and phenylalanine to that from control liver. The activity ratio of isoenzyme 1 preparation from control liver for these amino acids was not changed after electrophoresis and isoelectric focusing. Isoenzyme 1 preparations from liver of control and glucagon-treated rats had nearly identical physical and enzymic properties suggesting that both enzymes are the same protein, and that the increase in isoenzyme 1 in rats treated with glucagon may be accounted for as a net increase in a protein species already present in liver.

On the basis of response to glucagon, electrophoresis, isoelectric focusing, inhibition by common substrate and pH profile of isoenzyme 1, it is suggested that histidine–pyruvate aminotransferase is identical with phenylalanine–pyruvate aminotransferase.

The observations made with isoenzyme 2 were also entirely consistent with that suggestion. Isoenzyme 2 was not purified to the same extent, however, because of its low activity both in the mitochondria and in the cytosol.

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