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Isolation and Characterization of Multiple Forms of Glutamate-Aspartate Aminotransferase from Pig Heart

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SUMMARY

At least four different protein fractions provided with aspartate aminotransferase activity can be isolated from pig heart cytoplasm. No difference is detected in the sedimentation coefficient, immunodiffusion properties, and primary structure (as studied by quantitative amino acid analysis and peptide mapping of tryptic digests) of the three main fractions, called, respectively, α, β, and γ in their order of increasing anodic mobility on starch gel electrophoresis. The three main fractions differ significantly in specific activity, kinetics of recombination of the apoenzyme with the coenzyme, and behavior in 8 M urea. The most visible difference among them is in the way in which the coenzyme is bound to the enzyme protein. In the α and β fractions, pyridoxal phosphate is bound mostly in an "active" mode, characterized by absorption peaks at 362 or 430 mμ (depending upon the pH) and by the capacity to transaminate reversibly with the amino acid substrates. In the γ form, most of the coenzyme is bound in a nonactive mode, characterized by an absorption peak at 340 mμ and by the incapacity to react with amino acid substrates. Upon aging, the α and β fractions lose some activity, while a portion of their coenzyme becomes bound in the inactive mode. Even after these changes have occurred, the various fractions maintain their electrophoretic individuality, which is also preserved after resolution and after treatment with concentrated urea.

Electrophoretic analyses carried out on a water extract of a single heart taken immediately after the death of the animal reveal the presence of the various subforms; this indicates either that the various subforms exist as such in the living cell or, if they are artifacts, that they must be formed very early in the preparation and under extremely mild conditions.

The occurrence of multiple forms and isozymes has recently been reported for several enzyme systems (1). Glutamate-aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) has been isolated in large amounts and in a high degree of purity from pig heart (2, 3). The occurrence of two different enzymes provided with aspartate aminotransferase activity has been demonstrated in various biological materials, including different rat tissues (4, 5) and pig and human heart (6–9). The nature of the two forms of the enzyme, which some authors call "cytoplasmic" and "mitochondrial" and others call "anionic" and "cationic," remains controversial. Moreover, Wada and Morino (6), as well as Nisselbaum and Bodansky (8, 9), found differences in the kinetic behavior of the two forms of the enzyme, while Henson and Cleland (7) concluded that they were identical from this point of view. The possibility that each of these forms of aspartate aminotransferase might contain a mixture of subforms was pointed out by Decker and Rau (10) on the basis of experiments on crude cell extracts and on nonhomogeneous commercial preparations, and by Jenkins and Taylor (11), who studied highly purified preparations. Recently, we (12) have shown that each of the two isozymes contains at least three subforms and have obtained a partial separation of the three subforms of the cytoplasmic isozyme. The various subforms were found to differ in their specific activity and spectral properties.

In the present paper a new procedure is described for the isolation of the main subforms of cytoplasmic aspartate aminotransferase. The present procedure has the notable advantages over our previous method (12) of avoiding resolution of the enzyme during the purification (this is important because the apoenzyme is considerably less stable than the holoenzyme) and of leading to improved separation of the three main subforms. Evidence has been obtained suggesting that the main subforms are not artifacts produced during the purification.

The three main subforms have been characterized with respect to their physical (absorption spectra, optical rotatory dispersion, sedimentation coefficient), chemical (amino acid composition, coenzyme content, analysis of peptides obtained by partial digestion), and functional (substrate specificity, steady state kinetics, kinetics of recombination of apo- with coenzyme, kinetics of urea denaturation) properties.

These investigations have revealed both similarities and differences among the subforms.

EXPERIMENTAL PROCEDURE

Materials

Malate dehydrogenase and NADH were obtained from Boehringer; α-chymotrypsin, from Worthington; trypsin, from

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Seravac; α-ketoglutaric acid, from Fluka; pyridoxal 5′-phosphate, from Hoffmann-La Roche; fast violet B salt (diazotate 6-benzoylamino-4-methoxy-*m*-toluidine), from Sigma; amino acids, from Merck; hydrolyzed starch, from Connaught; carboxymethyl Sephadex C-50 (medium), from Pharmacia; and nigrosin, from C. Erba.

Methods

Assay of Enzymatic Activity—Glutamate-aspartate aminotransferase activity was determined at 25° as previously reported (12); keto acid analyses were carried out as described by Lis, Fasella, Turano, and Vecchini (13) and by Jenkins and Sizer (14). The steady state kinetic analysis was carried out essentially as described by Velick and Vavra (15), but the assay method was modified slightly by replacing arsenate buffer with 0.1 M Tris-HCl, pH 8.0, which, being a better catalyst of the enolization of oxalacetate, avoids the lag phase (16), and by using cuvettes with a 10-cm optical path.

Amino Acid Analyses—Quantitative amino acid analyses were performed in a Beckman/Spinco automatic amino acid analyzer after acid hydrolysis in evacuated sealed tubes according to Moore, Spackman, and Stein (17). Sulfhydryl group determinations were performed as described by Boyer (18). Tryptophan was estimated spectrophotometrically on the apoenzyme in 0.1 N NaOH (19). Ammonia was determined according to Hirs, Stein, and Moore (20).

Preparation of Apoenzyme—The holoenzyme was treated twice consecutively as described by Scardi, Scotto, Iaccarino, and Scarano (21) to dissociate the aminotransferase into apo- and coenzyme, and then was dialyzed extensively against 0.05 M acetate buffer, pH 5.4.

Recombination of Apoenzyme with Coenzyme—The apoenzyme at a concentration of 1.4×10^{-8} M (assuming a molecular weight of 47,000) in 5×10^{-2} M Tris-HCl buffer, pH 7.2, was incubated at 5° with pyridoxal phosphate at concentrations of 4×10^{-6} or 4×10^{-7} M. It is important to note that the concentration of Tris buffer used in these experiments was much lower than that required for the formation of Schiff bases with the carbonyl group of pyridoxal phosphate (22). Samples of 0.5 ml each were taken from the reaction mixture at various times of incubation and rapidly added to 2.5 ml of a solution containing potassium phosphate buffer, pH 7.4 (final concentration, 9.1×10^{-2} M), aspartate (final concentration, 5.7×10^{-2} M), NADH (final concentration, 8×10^{-5} M), and malate dehydrogenase (final concentration, 1.47 μg per ml). The samples were incubated in a water bath until their temperature had reached 25°, and 0.4 ml of 0.05 M potassium ketoglutarate was added to each sample. Immediately after this addition, the test solution was placed in the cuvette (1-cm optical path) of a Beckman DK-2 recording spectrophotometer (provided with a cell holder thermostated at 25°), and the decrease of optical density at 340 mμ consequent to the oxidation of NADH by the oxalacetate formed in the transamination reaction was recorded. The slope of the straight line recorded on the spectrophotometer is linearly related to the concentration of the active holoenzyme. Preliminary tests had shown that in the assay mixture the rate of recombination of the apoenzyme with pyridoxal phosphate is slowed down to a negligible value by the presence of the concentrated phosphate buffer and that no dissociation of the holoenzyme into pyridoxal phosphate and apoenzyme occurs.

Partial Hydrolysis—Solid urea (final concentration, 8 M) was added to a solution containing 40 to 50 mg of protein and 0.03 ml of mercaptoethanol in 10 ml of water. The pH was adjusted to 8 with sodium carbonate. A sodium iodoacetate solution was then added to give a final concentration of 15 mg per ml (23). The solution was kept for 10 min in the dark and then dialyzed against several changes of distilled water for at least 36 hours. The resulting suspension was heated at 100° for 3 min, and its pH was adjusted to 8 to 8.5 with 1 M ammonium bicarbonate. Trypsin was then added in an amount corresponding to 3.33% by weight of the protein to be digested.

The digestion was continued for 2½ hours at 37° and then stopped by lowering the pH to 5.4 by the addition of 1 M acetic acid. The small amount of insoluble material was collected by centrifugation; the supernatant was lyophilized to dryness, and the salts were removed by sublimation as described by Hirs, Moore, and Stein (24). The lyophilized material was dissolved in a few drops of water, and aliquots were spotted on several papers. The papers (Whatman No. 3MM, 33.5 × 35 cm) were developed by chromatography (isoamyl alcohol-pyridine-water, 35:35:29) for 10 hours, dried, and developed along the other dimension by electrophoresis with a pyridine-acetic acid-water (25:25:2950) buffer, pH 4.6 (25). A potential of 1300 volts, giving an intensity of 40 to 50 ma, was applied for 90 min. The dried papers were stained with 0.25% ninhydrin in acetone.

The material which remained insoluble after tryptic digestion was washed twice with water and digested with α-chymotrypsin under the conditions described above for the digestion with trypsin.

Spectrophotometric and Spectropolarimetric Measurements—Absorption spectra were measured in a Beckman DK-2A recording spectrophotometer. Optical rotatory dispersion measurements were performed in a Cary model 60 recording spectropolarimeter. Each sample was analyzed both at pH 8.0 (in 0.1 M Tris-HCl buffer) and at pH 5.0 (in 0.05 M sodium acetate buffer). The experimental details were the same as those previously described (26).

Ultracentrifugation—Sedimentation was followed by schlieren optics in a Spinco model E ultracentrifuge with an RTIC temperature control unit. All runs were made at 50,740 rpm in the temperature range of 14–16°. Sedimentation velocities were evaluated from the movement of the maximum ordinate of the schlieren peak, by a least squares procedure. Concentrations were taken as the average of those corresponding to the first and the last exposures measured. Values of the sedimentation coefficients were reduced in the conventional way to $s_{20,w}$.

Fractionation of Various Subforms of Cytoplasmic Aspartate Aminotransferase from Pig Heart—Two modifications of the procedure of Jenkins, Yphantis, and Sizer (2) were used to prepare the enzyme.

According to one procedure (Method A), the previously described (12) modification of the method of Jenkins *et al.* was followed up to the stage prior to chromatography on carboxymethyl Sephadex. According to the other procedure (Method B), the above modification of the method of Jenkins *et al.* was followed up to the fractionation with ammonium sulfate. The ammonium sulfate precipitate was dissolved in water and extensively dialyzed against sodium acetate buffer of ionic strength 0.02, pH 5.4, chromatographed on carboxymethyl cellulose, and precipitated with acetone as described by Lis (3). The precipitate was dissolved in a minimum amount of cold water, dialyzed overnight, and adjusted to pH 7.4 with potassium phosphate

buffer at a final concentration of 0.02 M. The enzyme solution was then placed on a DEAE-cellulose[1] column (3 × 20 cm) equilibrated with the same buffer. The column was washed with the same buffer, and the effluent was analyzed for proteins and aspartate aminotransferase activity. A single fraction with constant specific activity was recovered from the column. The active fractions were pooled and precipitated with 1 volume of cold acetone.

The enzyme (about 400 mg) obtained by either of the above procedures was extensively dialyzed against 0.02 M sodium acetate buffer, pH 5.33. The solution was then carefully layered on the top of a carboxymethyl Sephadex column (4 × 50 cm) equilibrated with the same buffer. The column was washed successively with 100 ml of 0.04 M and 800 ml of 0.06 M acetate buffer, pH 5.33. With the latter buffer, the first protein fraction is eluted from the column. A gradient obtained by placing 1000 ml of 0.06 M acetate buffer, pH 5.33, in the mixing chamber and 1000 ml of 0.11 M acetate buffer, pH 5.41, in the reservoir was then applied. All pH values were carefully determined in a Radiometer pH meter, model 4.

The elution pattern of a typical chromatographic separation is shown in Fig. 1. Each protein fraction was collected as indicated by the *arrows* in Fig. 1, and concentrated by ultrafiltration or by precipitation with an equal volume of cold acetone. The concentrated fractions were subjected to electrophoresis on starch gel according to Martinez-Carrion and Jenkins (27). After the run the gel was sliced horizontally; the lower layer was stained for proteins with nigrosin, and the upper one was tested for aspartate aminotransferase activity according to Decker and Rau (10).

Immunological Studies[2]—Ten milligrams of protein from chromatographic Fraction IV were injected intravenously into a rabbit weighing 3 kg. A month after the first injection, the rabbit received another 5 mg of the same material. A week later the rabbit was bled; the blood was collected, and the antiserum was prepared and stored at −20°. The protein from each of the three main chromatographic fractions was then separately tested according to Ouchterlony (28) against the rabbit antiserum thus obtained.

RESULTS

Purification and Spectral Properties of Cytoplasmic Aspartate Aminotransferase Subforms—The preparation obtained by Method A (Fig. 2) contains some inactive contaminants which are not present in the preparation obtained by Method B (Fig. 3). The latter method, however, gives about 20% lower yield. Figs. 2 and 3 also show that both preparations contain at least four distinct electrophoretic fractions, all of which display some enzymatic activity (Fig. 4). These four main electrophoretic fractions were called α, β, γ, and δ in the order of their increasing anodic mobility.

Chromatography on carboxymethyl Sephadex under the conditions described above separated the cytoplasmic aspartate aminotransferase preparations obtained by either Method A or Method B into four fractions (see Fig. 1). The first fraction (hereafter indicated as I) contains Subforms γ and δ (see Fig. 3) and, when the starting material has been obtained by Method A, also contains minor components which on starch gel electrophoresis

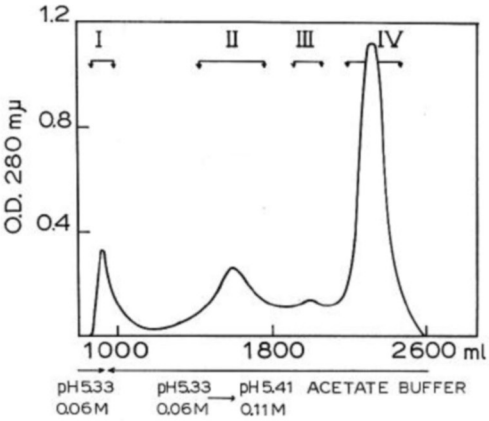

FIG. 1. Chromatography on carboxymethyl Sephadex C-50 of pig heart cytoplasmic aspartate aminotransferase prepared according to Method A. The sequence of the buffers is indicated in the figure. For further details, see the text.

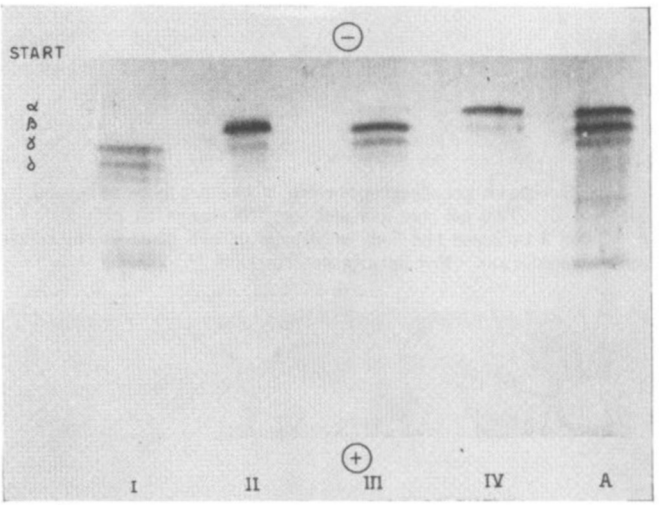

FIG. 2. Starch gel electrophoresis of the proteins present in the preparation obtained by Method A (*A*) and in chromatographic Fractions I, II, III, and IV. The gel was stained for proteins with nigrosin. α, β, γ, and δ indicate the four subforms of cytoplasmic aspartate aminotransferase. For details, see the text.

migrate to the anode ahead of the active bands. The second and third fractions (hereafter indicated, respectively, as II and III) contain Subform β and evident traces of Subform γ. The fourth fraction (IV) contains essentially Subform α with traces of Subform β. It is important to observe that, since the impurities present in the preparation obtained by Method A essentially migrate with Fraction I, Method A is preferable, because of its higher yield, if the main object of the preparation is to obtain large amounts of Fractions III and IV.

The absorption spectra in the 300 to 500 mμ range of the four chromatographic fractions at pH 5.4 are shown in Fig. 5. The first chromatographic fraction contains a large amount of material absorbing at 340 mμ; the second and third chromatographic fractions have more of the 430 mμ- and less of the 340 mμ-absorbing material, while the fourth chromatographic fraction contains only material with an absorption maximum at 430 mμ.

The relative amounts of the material absorbing at 340 mμ and at 430 mμ present in each chromatographic fraction were evalu-

[1] A. J. Lawrence, personal communication.

[2] We are grateful to Dr. E. Bucci for performing the immunological tests.

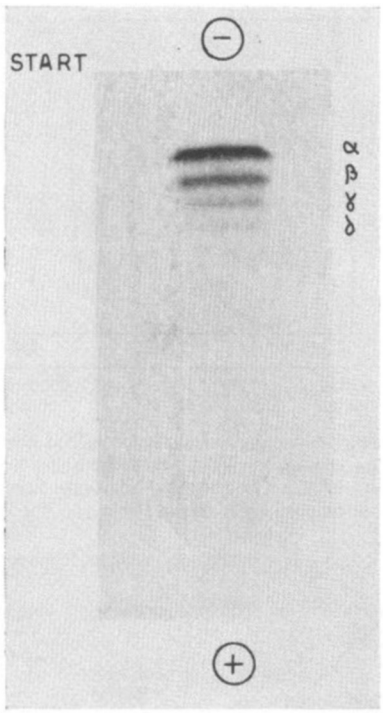

FIG. 3. Starch gel electrophoresis of the proteins obtained by Method B. The gel was stained for proteins with nigrosin. α, β, γ, and δ indicate the four subforms of cytoplasmic aspartate aminotransferase. For details, see the text.

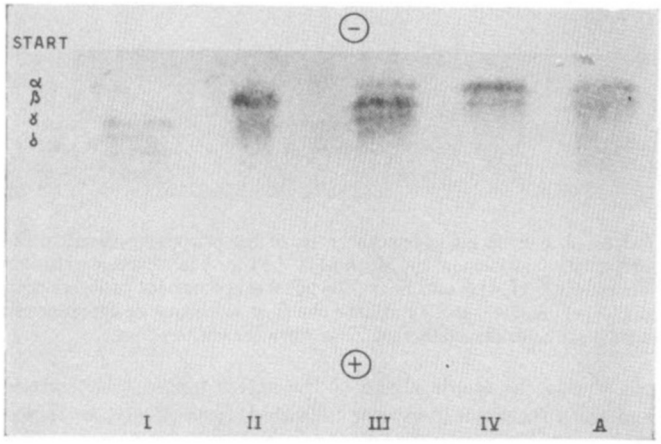

FIG. 4. Starch gel electrophoresis of the proteins present in the preparation obtained by Method A (*A*) and in chromatographic Fractions I, II, III, and IV. The gel was stained for aspartate aminotransferase activity according to Decker and Rau (10).

ated with the use of the respective extinction coefficients previously determined (12). The results of these computations, and the specific activities of the various chromatographic fractions, are reported in Table I. The specific activity of the various chromatographic fractions is inversely proportional to the relative amount of material with an absorption maximum at 340 mμ present in each fraction; this is in agreement with our previous conclusion, based on the spectral properties in the presence and absence of substrates, that the material absorbing at 340 mμ is enzymatically inactive.

The quantitative agreement between the experimental data and the assumption that the material with an absorption maximum at 340 mμ is fully inactive is clearly evident in the last column of Table I, which gives the corrected specific activities of the various fractions, obtained by dividing the experimentally determined specific activity by the fraction of the total material computed to be in the active form. As shown in Table I, the values for corrected specific activity are very similar for all fractions, except for Fraction I, which has somewhat lower values, probably because it contains some impurities not related to transaminase.

Since, as shown in Table I and Figs. 2 and 4, chromatographic Fraction I essentially corresponds to the electrophoretic Subform γ, it seems obvious that this subform has a lower activity, and a corresponding higher content of inactive material absorbing at 340 mμ, than the other subforms.

Removal of Coenzyme from Protein—The spectra of the four fractions after they had been treated as described by Scardi *et al.* (21) for preparing the apoenzyme are shown in Fig. 6. It is evident that after this treatment none of the fractions exhibits any absorbance at 430 mμ. On the other hand, the amount of

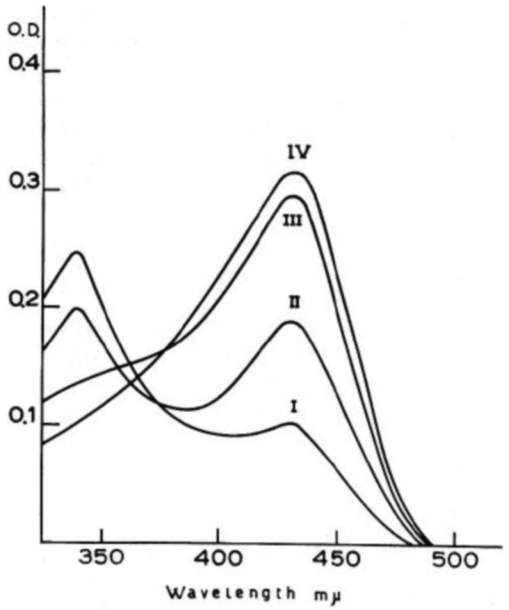

FIG. 5. Absorption spectra of chromatographic Fractions I, II, III, and IV. All spectra were recorded in 0.06 M sodium acetate buffer, pH 5.4, at a protein concentration of 2.7 mg per ml.

TABLE I

Relationship between specific activity and relative amounts of material absorbing at 340 mμ

Fraction and electrophoretic band	Material absorbing at 340 mμ	Specific activity[a]	Corrected specific activity
	%		
I. γ, δ	68	24	75
II. β, γ	40	69	115
III. β, traces of γ	11	98	110
IV. α, traces of β	0	119	119

[a] Expressed as micromoles of oxalacetate produced per min per mg of protein under the assay conditions given in the text.

material with an absorption maximum at 340 mμ found in each fraction after this treatment corresponds to that previously calculated from the spectra recorded before the treatment (Fig. 5).

In a previous paper (12) we have shown that pyridoxal phosphate can be detached from the material absorbing at 340 mμ, as well as from the material absorbing at 430 mμ, by treatment with 0.1 M NaOH; this treatment, however, denatures the protein. It seems clear, therefore, that both the absorption peak at 340 mμ and the one at 430 mμ are due to protein-bound pyridoxal phosphate, but that pyridoxal phosphate is bound much more tightly in the form absorbing at 340 mμ than in the form absorbing at 430 mμ.

The starch gel electrophoretic patterns of the main chromatographic fractions were not significantly modified after the removal of the coenzyme absorbing at 430 mμ, except that the mobility of all fractions was slightly increased. This indicates that the difference among the subforms persists even after removal of the "active" coenzyme.

Recombination of Apoenzyme with Coenzyme—The proteins from Fractions II and IV, which had been subjected to the mild treatment for the removal of the "active" coenzyme (21), were used to study the recombination with pyridoxal phosphate. The protein from Fraction I was not used for this investigation because it consists largely of the inactive material absorbing at 340 mμ, which cannot be resolved without denaturation.

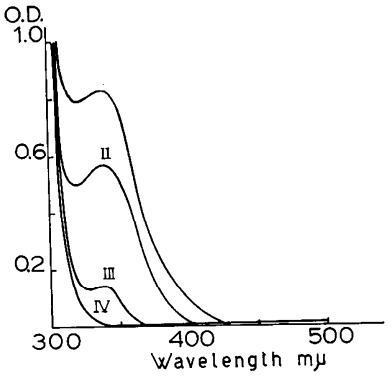

FIG. 6. Absorption spectra of Fractions I, II, III, and IV after treatment for detaching the active coenzyme (21). All spectra were recorded in 0.06 M sodium acetate buffer, pH 5.4, at a protein concentration of 10 mg per ml.

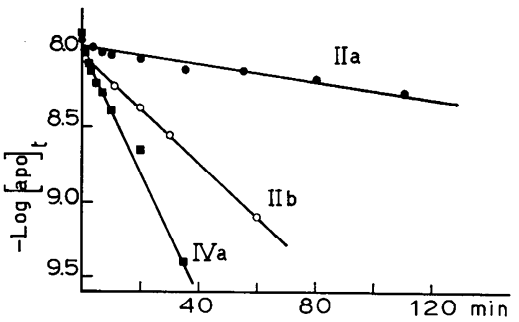

FIG. 7. First order plot of the recombination reaction of 1.4×10^{-8} M apoenzyme (*apo*) from Fractions II and IV with pyridoxal phosphate at a concentration of 4×10^{-7} M (*IIa* and *IVa*) and 4×10^{-6} M (*IIb*). The values of the bimolecular rate constants calculated from each experiment and expressed in M^{-1} sec^{-1} were 1.6×10^4 for *IIa*, 1×10^4 for *IIb*, and 2.3×10^5 for *IVa*. The experimental details and elaboration of the data are described in the text.

TABLE II

Values of K_m for Fractions II and IV of aspartate aminotransferase

Enzyme fraction	K(L-aspartate)	K(α-ketoglutarate)
	M	M
II	2.0×10^{-3}	1.5×10^{-4}
IV	2.4×10^{-3}	1.4×10^{-4}

TABLE III

Half-time rates and extent of transamination reaction between various amino acids and aldehydic form of main chromatographic fractions of cytoplasmic aspartate aminotransferase

Amino acid and concentration	Fraction I		Fraction II		Fraction IV	
	$t_{\frac{1}{2}}$	Extent[a]	$t_{\frac{1}{2}}$	Extent[a]	$t_{\frac{1}{2}}$	Extent[a]
	sec	%	sec	%	sec	%
DL-Alanine, 0.2 M	75	31	80	60	70	100
DL-Methionine, 0.1 M			5	60	5	100
DL-Serine, 0.14 M	200	32	205	60	205	100
DL-Valine, 0.14 M						0
Glycine, 0.14 M						0
L-Leucine, 0.07 M			150	60	135	100

[a] The extent of transamination is expressed as the percentage of pyridoxal phosphate molecules bound to aspartate aminotransferase which have undergone transamination upon completion of the reaction.

The results of typical experiments are reported in Fig. 7. As shown in the figure, under the experimental conditions used, the recombination of the apo- and coenzyme follows first order kinetics until more than 90% complete, as expected for a bimolecular reaction in the presence of an excess of one of the substrates, *i.e.* pyridoxal phosphate. The bimolecular rate constant for recombination can be calculated by dividing the values for the pseudomonomolecular rate constants (which are given by the slope of the first order plots in Fig. 7) by the concentration of pyridoxal phosphate in the corresponding experiment; the latter value remains practically constant throughout the reaction, since the initial concentration of pyridoxal phosphate is always at least 25 times greater than that of the apoenzyme. As shown in Fig. 7, the values of the bimolecular recombination rate constant for chromatographic Fraction II calculated from the data obtained at the two concentrations of the coenzyme agree within experimental error. A comparison between the recombination rates of Fractions II and IV of the transaminase with 4×10^{-7} M pyridoxal phosphate shows that Fraction IV recombines with pyridoxal phosphate at least one order of magnitude faster than Fraction II (Fig. 7).

Reaction with Substrates—The behavior of the three chromatographic fractions of aspartate aminotransferase toward the substrates was studied at equilibrium by spectrophotometric analyses and kinetically under steady state conditions.

The results of spectrophotometric studies at equilibrium essentially confirm those previously published in our preliminary note (12), and can be briefly summarized as follows.

The protein from chromatographic Fraction IV, corresponding to Subform α, has a single absorption maximum above 300 mμ:

TABLE IV

Amino acid composition of chromatographic fractions of aspartate aminotransferase

Hydrolysis times were 24, 42, and 52 hours at 110°. Except when otherwise indicated, the values represent the average of determinations at different hydrolysis times. The variation is expressed as the average deviation. Only serine undergoes appreciable destruction from 24 to 52 hours; the reported values are extrapolated to zero time. In some instances an increase in the amino acid was found with longer hydrolysis times. In these cases only the value at 52 hours is used.

Amino acid	Fraction I			Fraction II			Fraction III			Fraction IV		
	Residues	Calculated No. of residues for mol wt 47,000	Nearest integer	Residues	Calculated No. of residues for mol wt 47,000	Nearest integer	Residues	Calculated No. of residues for mol wt 47,000	Nearest integer	Residues	Calculated No. of residues for mol wt 47,000	Nearest integer
	g/100 g protein			*g/100 g protein*			*g/100 g protein*			*g/100 g protein*		
Lysine	5.30 ± 0.09	19.4	19	5.40 ± 0.19	19.8	20	5.23 ± 0.17	19.2	19	5.38 ± 0.19	19.7	20
Histidine	2.23 ± 0.03	7.7	8	2.29 ± 0.06	7.9	8	2.21 ± 0.06	7.6	8	2.26 ± 0.01	7.7	8
Arginine	8.61[a]	25.9	26	8.82[a]	26.6	27	8.57[a]	25.8	26	8.66[a]	26.1	26
Aspartic acid	10.46 ± 0.06	42.7	43	10.37 ± 0.07	42.4	42	10.35 ± 0.06	42.3	42	9.90 ± 0.23	40.5	41
Threonine	5.14 ± 0.01	23.9	24	5.07 ± 0.07	23.6	24	5.09 ± 0.04	23.7	24	5.13 ± 0.05	23.8	24
Serine	4.49[b]	24.2	24	4.47[b]	25.6	26	4.79[b]	25.9	26	4.88[b]	26.3	26
Glutamic acid	12.47 ± 0.04	45.4	45	12.67 ± 0.10	46.2	46	12.67 ± 0.05	46.2	46	12.58 ± 0.01	45.8	46
Proline	4.94 ± 0.06	23.9	24	5.16 ± 0.05	25.0	25	4.97 ± 0.07	24.1	24	4.89 ± 0.16	23.7	24
Glycine	3.37 ± 0.02	27.8	28	3.42 ± 0.02	28.0	28	3.46 ± 0.03	28.6	29	3.44 ± 0.01	28.4	28
Alanine	4.90 ± 0.03	32.4	32	4.89 ± 0.04	32.4	32	4.88 ± 0.05	32.3	32	4.97 ± 0.00	32.9	33
Valine	5.92[a]	28.1	28	5.74[a]	27.3	27	6.03[a]	28.6	29	6.02[a]	28.6	29
Methionine	1.67 ± 0.06	6	6	1.58 ± 0.04	5.6	6	1.58 ± 0.01	5.7	6	1.63 ± 0.03	5.8	6
Isoleucine	4.31[a]	17.9	18	4.31[a]	17.9	18	4.41[a]	18.3	18	4.39[a]	18.2	18
Leucine	9.52[a]	39.5	40	9.41[a]	39.1	39	9.65[a]	40.1	40	9.67[a]	40.2	40
Tyrosine	4.17 ± 0.03	12.0	12	4.09 ± 0.14	11.8	12	4.02[c]	11.6	12	4.04 ± 0.03	11.7	12
Phenylalanine	7.30 ± 0.04	23.3	23	7.07 ± 0.07	22.6	23	7.08[c]	22.7	23	7.24 ± 0.02	23.1	23
Tryptophan	4.21[c]	10.6	11	3.94[c]	10.0	10	3.96[c]	10.0	10	3.91[c]	9.9	10

[a] Fifty-two-hour value only.
[b] Extrapolated value.
[c] Single determination.

TABLE V

Sulfhydryl groups and ammonia determination on the four main chromatographic fractions of cytoplasmic aspartate aminotransferase

Fraction	Residues found for mol wt 47,000		
	—SH in water	—SH in 8 M urea	NH_3
I	3.4	3.8	38
II	3.0	3.9	41
III	3.3	3.9	39
IV	2.9	4.2	41

the position of the maximum depends upon the pH (362 mμ at alkaline pH, 430 mμ at acidic pH), the pK for the transition being 6.3. As observed by previous authors (29, 30), the yellow, protonated form reacts with the keto acid substrates to give abortive complexes, while the colorless, nonprotonated form reacts reversibly with glutamate or aspartate to give an aminic form of the enzyme with an absorption peak at 333 mμ and the corresponding keto acid (13, 14). The association constant for the complex between ketoglutarate and aminic enzyme is relatively high (2×10^3 M^{-1}) (31). The material absorbing at 430 or 362 mμ present in chromatographic Fractions II and III behaves similarly.

The material absorbing at 340 mμ, which is present in variable amounts in Fractions II and III and constitutes the main part of Fraction I, is not modified either by variation of pH between 4.5 and 9 or by addition of keto acid or amino acid substrates. Moreover, no evidence for the formation of keto acids after incubation of this material with either aspartate or glutamate was found. These results indicate that the material absorbing at 340 mμ is unable to react with the substrates of aspartate aminotransferase, in agreement with the results of specific activity determination (Table I).

The steady state kinetic behavior of all subforms was consistent with the shuttle mechanism (15) predicted by the hypothesis of Snell (Guirard and Snell (32)) and Braunstein (33). The values of the kinetic parameters for Subforms α (Fraction IV) and β (Fraction II) are reported in Table II. The values are essentially the same for the two cytoplasmic subforms and are in fair agreement with those reported by previous authors (7–9, 15, 16). A kinetic investigation of the subforms of mitochondrial aspartate aminotransferase (34) revealed notable differences between the mitochondrial and cytoplasmic subforms, in agreement with the results of Wada and Morino (6) and Nisselbaum and Bodansky (9).

Substrate Specificity—The reaction between various amino acids and aspartate aminotransferase was followed as described by Lis *et al.* (13). Solutions of Fractions I, II, and IV, containing 5 mg of the phosphopyridoxal form of the enzyme per ml in 0.1 M Tris-HCl buffer, pH 8.0, were incubated with the test amino acid at the indicated concentration.

The spectra of the resulting mixtures were recorded in a Beckman DK-2A spectrophotometer with a cell holder thermostated at 25°, at various times after the addition of the amino acid. When transamination between enzyme and amino acid occurs, the absorption maximum of the solution in the near ultraviolet region shifts from 362 to 333 mμ, and chemical determinations show the presence of the corresponding keto acid in the reaction mixture.

With glutamate and aspartate the reaction is quite fast. Measurements carried out with the stopped flow method (35, 36) showed that the time required for the spectral change to reach 50% completion under the above conditions is of the order of a few milliseconds.

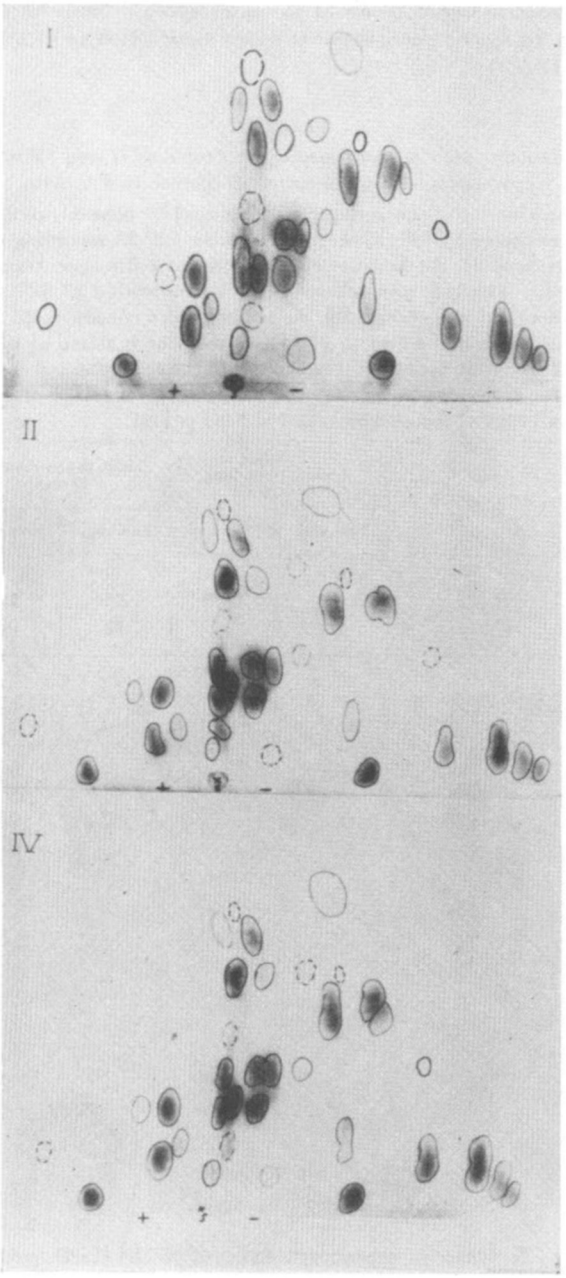

FIG. 8. Peptide maps of the tryptic hydrolysate of Fractions I, II, and IV of cytoplasmic aspartate aminotransferase. For details, see the text.

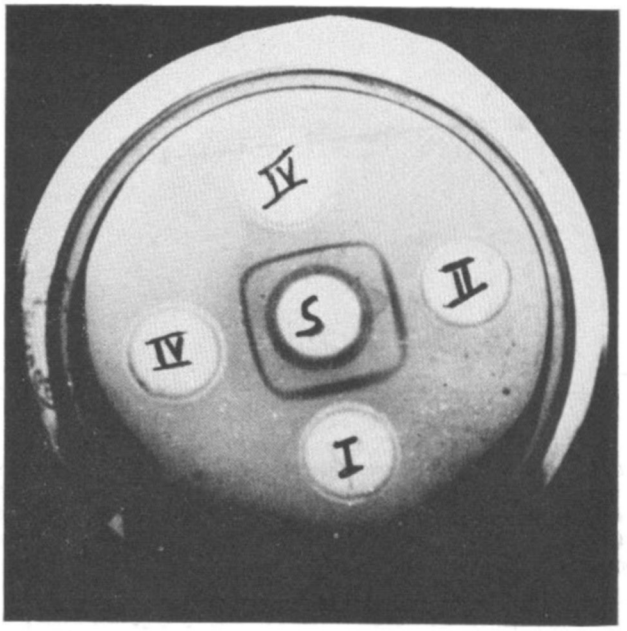

FIG. 9. Double diffusion on agar gel. Antiserum to Fraction IV of pig heart cytoplasmic aspartate aminotransferase was placed in the *central well* (*S*); the protein from chromatographic Fractions I, II, and IV was placed in the *lateral wells* as indicated in the figure.

The results obtained with several other amino acids are reported in Table III. As shown in the table, no appreciable difference in specificity was noted between the fractions. The rate of the reaction becomes progressively slower as the structural differences between the amino acid substrates and aspartate or glutamate increase.

Chemical Composition—The coenzyme content of the three main fractions was measured as described by Lis *et al.* (13) and Jenkins and Sizer (14) and was found, for all fractions, to be 1 mole of coenzyme per 47,000 ± 3,000 g of protein.

Amino acid analysis of the three chromatographic fractions of cytoplasmic aspartate aminotransferase gave the results reported in Table IV.

The results of sulfhydryl group and amide nitrogen determinations are reported in Table V.

Typical peptide maps of the tryptic hydrolysates of Fractions I, II, and IV are shown in Fig. 8. With all fractions the same 38 peptides appeared on the electrophoretograms. Similarly, the peptides obtained by chymotryptic digestion of the material which remained insoluble after tryptic digestion did not reveal any difference among the various fractions.

Immunological Studies—As shown in Fig. 9, double diffusion on agar gel of the three main fractions of the cytoplasmic enzyme against the serum of a rabbit immunized with the α subform gave only one precipitation line. This suggests that, within the limits of the method, no immunological difference can be detected among the subforms.

Ultracentrifugation—The sedimentation coefficients ($s_{20,w}$) of the chromatographic Fractions I, II, and IV are reported in Fig. 10 as a function of protein concentration. Both at pH 7.50 and 5.38 the concentration dependence of $s_{20,w}$ for Fractions I and II

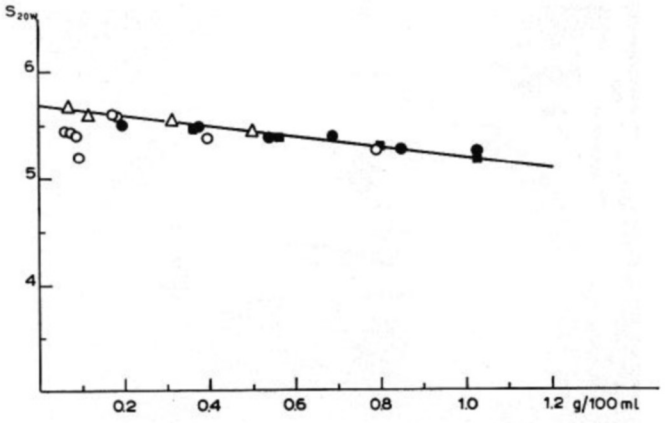

FIG. 10. Concentration dependence of the sedimentation coefficients of the protein from Fractions I, II, and IV. △, Fraction I in 0.09 M potassium phosphate, pH 7.5; ○, Fraction IV in the same buffer; ●, Fraction IV in 0.05 M sodium acetate, pH 5.38; ■, Fraction II in the same buffer. The *solid line* is the least squares fit calculated for Fractions I and II.

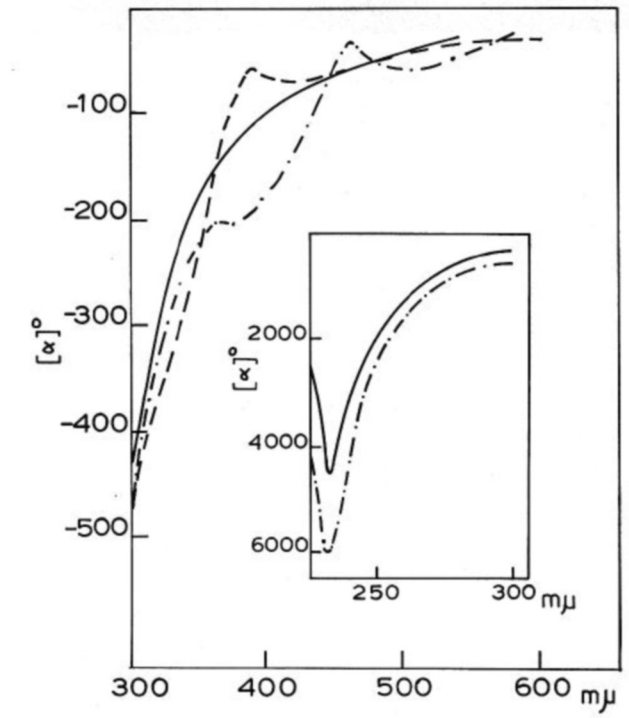

FIG. 11. Optical rotatory dispersion of the main chromatographic fractions of cytoplasmic aspartate aminotransferase. ——, Fraction I in 0.2 M sodium acetate buffer, pH 5.4, after treatment for detaching the active coenzyme; – – –, Fraction II (and IV) in 0.1 M Tris-HCl buffer, pH 8.0; –·–·, Fraction II (and IV) in 0.2 M sodium acetate buffer, pH 5.0.

follows a straight line with a negative slope. The values for Fraction IV are on the same line down to a protein concentration of about 0.2 g/100 ml, and fall below this straight line at lower concentrations.

Optical Rotatory Dispersion—Typical optical rotatory dispersion curves of chromatographic Fractions I, II, and IV are shown in Fig. 11. The material from Fractions II and IV was examined without further treatment, while that from Fraction I was analyzed before and after treatment for the removal of the "active" coenzyme according to Scardi *et al.* (21). The latter treatment gives material with an absorption maximum at 340 mμ and devoid of absorption at 430 and 360 mμ, and is therefore suitable for studying the possible occurrence of Cotton effects centered around 340 mμ.

Denaturation in Concentrated Urea and Aging—The rate of the structural modifications undergone in 8 M urea (37, 33) by the enzyme from Fractions II and IV was followed spectrophotometrically by recording the shift of the spectral peak from 430 to 330 mμ which occurs upon incubation of the enzyme in 8 M urea buffered at pH 5.0 by 0.1 M sodium acetate (37).

With both Fractions II and IV the spectral changes follow a biphasic curve (37). As shown in Table VI, the first phase of the reaction occurs at about the same speed in both fractions, while the second phase is slower with Fraction II than with Fraction IV.

TABLE VI

Denaturation of chromatographic Fractions II and IV of cytoplasmic aspartate aminotransferase in 8 M urea

The kinetics of the denaturation induced by concentrated urea was measured by following, in a Beckman DK-2A recording spectrophotometer, the decrease of absorption at 430 mμ occurring in 8 M urea. The cell compartment was thermostated at 30° ± 1°. The reaction was started by the addition of a concentrated solution of enzyme in water to a 9 M urea solution buffered at pH 5.0 by 0.1 M sodium acetate. The volume of the concentrated enzyme solution added was such that the final concentration of urea was 8 M and that of the enzyme was 3 to 4 mg per ml.

Fraction	Apparent half-time		Change in absorbance at 430 mμ	
	Fast step	Slow step	Fast step	Slow step
	min	*min*	%	%
II	35	1700	30	70
IV	35	140	10	90

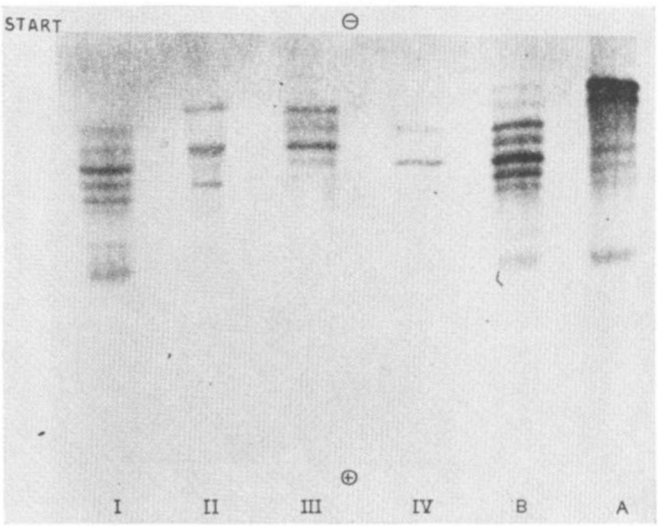

FIG. 12. Effect of exposure to maleate on the starch gel electrophoretic pattern of cytoplasmic aspartate aminotransferase. *A*, enzyme prepared according to Method A; *B*, *A* after exposure to maleate: *I*, *II*, *III*, and *IV*, chromatographic fractions after exposure to maleate. For details, see the text.

To study the effect of urea denaturation on electrophoretic behavior, the protein from the various fractions was kept in 8 M urea (containing 10^{-3} M mercaptoethanol) for 48 hours at 25°, and then analyzed by zonal electrophoresis on a starch gel prepared with 8 M urea as described by Smithies (39). Under these conditions the three main electrophoretic bands maintain their respective positions and no new band appears.

Similarly, aging of the enzyme (up to 40 days at 4° in 0.1 M potassium phosphate buffer, pH 6) did not produce marked changes in the electrophoretic pattern of the subforms. The spectrum recorded after aging, however, showed a slight but significant decrease of absorbance at 360 or 430 mμ, an increase at 340 mμ, and a parallel loss of specific activity. After 4 months of storage under the conditions described above, the protein from Fraction IV, which initially contained only the α subform, also exhibited, when analyzed by starch gel electrophoresis, some material with greater anodic mobility. Too little of the latter material was obtained for analysis to be attempted.

Artificial Production of Subforms by Treatment with Maleate—Exposure of the proteins from the various chromatographic fractions to 0.3 M sodium maleate, pH 6, for 5 min at 60° generates many new electrophoretic bands (Fig. 12).

Occurrence of α, β, and γ Subforms in Fresh Homogenates and during Purification Procedure—In order to minimize the manipulation undergone by the enzyme before the analyses and to reduce the probability of producing artifacts, a heart taken from a freshly killed pig was rapidly homogenized in cold water. The homogenate was centrifuged at 20,000 × *g* and the supernatant was analyzed by starch gel electrophoresis as previously described. The gel was stained for proteins and for transaminase activity. Less than 90 min elapsed between the death of the animal and the beginning of the electrophoretic run. The results in Figs. 13 and 14, *Pattern 1*, show that at least two distinct activity bands moving toward the anode and at least two bands moving toward the cathode were present in the homogenate. The latter group of bands corresponds to the mitochondrial system (5, 6). The two anodic bands correspond to the α and

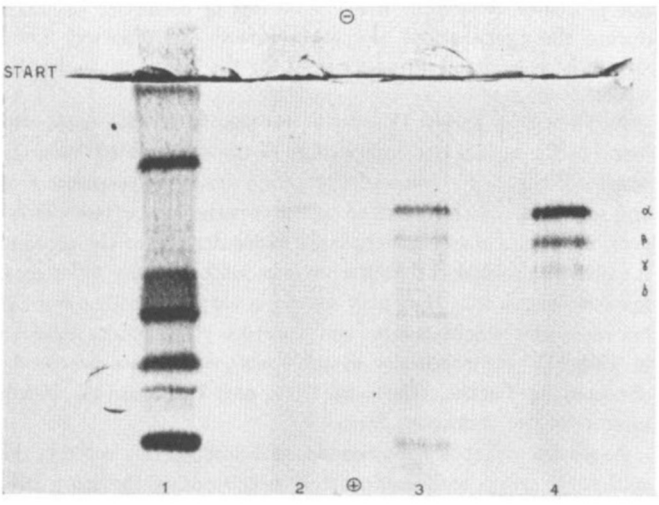

FIG. 13. Starch gel electrophoresis of the aminotransferase at various stages of purification; the gel was stained for proteins with nigrosin. *Pattern 1*, pig heart homogenate in water; *2*, after the heating step; *3*, after ammonium sulfate fractionation; *4*, preparation obtained by Method B. For details, see the text.

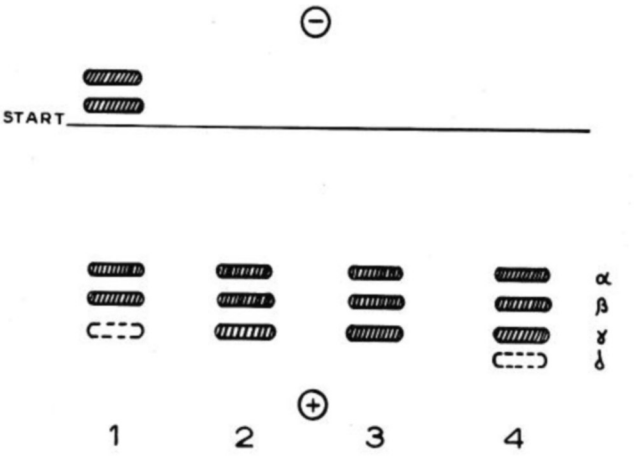

FIG. 14. Starch gel electrophoresis of the aminotransferase at various stages of purification; the gel was stained for aspartate aminotransferase activity as described elsewhere (10.) *Pattern 1*, pig heart homogenate in water; *2*, after the heating step; *3*, after ammonium sulfate fractionation; *4*, preparation obtained by Method B. For details, see the text.

β subforms of cytoplasmic aspartate aminotransferase described in this paper.

If the electrophoretic analysis is carried out after the first purification steps (heat treatment according to Jenkins *et al.* (2) as modified by Turano, Giartosio, Riva and Fasella (40)), the cationic bands disappear (Figs. 13 and 14, *Pattern 2*). After ammonium sulfate fractionation the anionic bands corresponding to the α and β subforms become much more marked, and two faster moving bands, corresponding to the γ and δ subforms, appear (*Pattern 3*). It seems possible that the two latter bands are present in the extract from the start, but, being weak, cannot be detected until a substantial concentration is achieved. This interpretation is consistent with the finding that in the electrophoretic analyses carried out after each purification step, down to the final purification (*Pattern 4*), the two slower anionic bands (α and β) are much larger than those of the two faster subforms (γ and δ).

DISCUSSION

It is evident from the results reported above that preparations of cytoplasmic aspartate aminotransferase previously considered to be homogeneous contain at least four distinct fractions, all provided with varying degrees of transaminase activity and separable by starch gel electrophoresis and ion exchange chromatography.

The correspondence between the electrophoretic and chromatographic properties of the various fractions is straightforward with the exception of Fraction III; its electrophoretic pattern is undistinguishable from that of Fraction II except for the presence of some traces of Subform α, which might account for the small spectral difference between Fractions II and III. It is not possible, at present, to tell whether Fraction III is a chromatographic artifact or is due to the existence of different types of the β subform which are not distinguishable by starch gel electrophoresis but separate on the chromatographic column. Further studies on this problem were not possible because too little of the material in Fraction III was obtained.

A comparison between the present fractionation of cytoplasmic

aspartate aminotransferase into subforms and that reported by other authors (9) would not be significant since the enzyme preparation used by them had been exposed to maleate, which, as shown by present results, may cause the formation of artificial subforms.

A comparison of the functional properties of the subforms shows that they all catalyze the same reaction by the same mechanism and have the same substrate affinity and specificity (see Tables II and III). The specificity of both the more active subforms corresponds to that established by previous authors for the mixture (41, 42). The similarity in the kinetic behavior of the two main subforms of cytoplasmic transaminase reported in this paper confirms the previously proposed (9, 12) interpretation of the discrepancy between the results of Henson and Cleland (7) and those of other authors (6, 8), relative to the occurrence of differences in the kinetic behavior of "anionic" and "cationic" aspartate aminotransferase.

The Michaelis constants for the substrates are the same for Fractions II and IV; it seems probable, therefore, that the difference in the specific activity (Table I) is related to the proportion of catalytically active molecules present in each fraction. This interpretation is supported by spectral studies; the specific activity of each fraction is, in fact, inversely proportional to the relative amount of material with an absorption maximum at 340 mμ which it contains. Equilibrium studies of the reaction of the various fractions with several amino acids show that the material absorbing at 340 mμ is unable to react either with the real substrates (aspartate and glutamate) or with other amino acids. This finding could explain the failure of Evangelopoulos and Sizer (43, 44) to obtain stoichiometric yields in the transamination between glutamate (or aspartate) and an enzyme preparation containing a large proportion of material absorbing at 340 mμ.

The enzymatic activity which all chromatographic fractions display both in solution and on starch gel electrophoresis is due to the amount of material with an absorption maximum at 430 mμ (at pH 5.4) which they all contain, although in varying amounts. Available evidence indicates that the material absorbing at 340 mμ contains pyridoxal phosphate bound in an "inactive" way. The position of the absorption maximum and the finding that $NaBH_4$ (12), carbonyl reagents (12), and mild resolving treatment (21) do not affect the binding, while concentrated acids and bases do so (12), suggest that pyridoxal phosphate is attached to the protein as a substituted aldimine derivative, possibly of the type proposed by Fischer, Forrey, Hedrick, Hughes, Kent, and Krebs (45) for phosphorylase at neutral pH. In the "active" subforms, with absorption peaks at 430 or 362 mμ, the coenzyme is instead found as a nonsubstituted aldimine (2, 33). The finding that, upon aging in the cold for 1 month or more, the α and β subforms exhibit some spectral changes (decrease of absorbance at 430 mμ and parallel increase of absorbance at 340 mμ), yet retain their characteristic electrophoretic mobility, suggests that, possibly as a consequence of partial denaturation or conformational change, the coenzyme may also become bound to the protein in an "inactive" mode in the α and β subforms.

In this respect the main difference between the α and β subforms, on one hand, and the γ subform, on the other, is that in the latter a large proportion of the coenzyme is bound "inactively" very early in the preparation, or possibly even within the cell.

Although both the α and β subforms can be reversibly resolved, the rate at which the apoenzyme recombines with pyridoxal phosphate is faster with the α than with the β subform. This difference should be kept in mind when interpreting the results of recombination kinetic studies on enzyme preparations containing several subforms (16, 46).

In general, the occurrence of enzymatic subforms which give functionally identical holoenzymes, but react with the coenzyme at different rates, could play a role in the adaptation of cellular enzyme systems to changes in the intracellular availability of pyridoxal phosphate.

The finding that the various subforms maintain their electrophoretic and chromatographic individuality after the "active" coenzyme has been removed by resolution indicates that the apoproteins of the various subforms are different and excludes the possibility that the subforms may be produced only by different modes of binding of the coenzyme to the same protein.

The available data on the chemical composition of the protein portion of the main fractions do not reveal any significant difference. The amount of protein which binds to 1 mole of coenzyme is essentially the same (47,000 ± 3,000). The value of 47,000 was assumed as the minimum molecular weight in the calculations of the amino acid composition of the protein. This figure is noticeably different from that reported previously for less pure preparations (2, 13). It is important to remember, however, that the value for the minimum molecular weight calculated from the coenzyme content may be higher or lower than the actual value. Errors of excess can in fact be produced by nonspecific binding of pyridoxal phosphate to the enzyme protein (47), while errors of defect can be caused by partial dissociation of the coenzyme from the protein during the preparation. In the present case, the occurrence of nonspecific binding seems highly improbable in view of the spectral properties of the enzyme and the fact that the enzyme was not exposed to pyridoxal phosphate during or after the preparation. The occurrence of extensive reversible resolution can be excluded, since the specific activity of the enzyme does not increase after incubation with pyridoxal phosphate. On the other hand it is possible, although not probable, that some irreversible loss of coenzyme occurred during the preparation. For this reason the reported figure should be considered an upper limit for the minimum molecular weight.

As shown in Tables IV and V, no significant difference was found in the amino acid composition of the various fractions; the small differences for some of the amino acids (*e.g.* aspartic acid and serine) are within or close to the experimental error. However, in view of the relatively large molecular size of the protein, it cannot be excluded that the various subforms may differ by a few residues, or that they may contain a varying small amount of low molecular weight, nonprotein material. The results reported in Table IV are essentially in agreement with those previously obtained by Turano, Giartosio, Riva, and Vecchini (48) with a mixture of the various subforms.

As shown in Table V, no significant difference was noted in the sulfhydryl group and amide nitrogen content of the main subforms. The number of —SH groups present in 47,000 g of protein (Table V) differs by 1 unit from the value previously reported (48) for a mixture of the three subforms prepared according to Lis (3). The discrepancy can be explained by the fact that aging causes the loss of one —SH group (48) and that

the preparation of the subforms requires a longer time than the method of Lis.

The great similarity in the primary structure of the subforms is confirmed by the identity of the peptide maps obtained after tryptic hydrolysis (Fig. 8). Thirty-eight peptides can be identified on the electrophoretic-chromatographic maps obtained after tryptic digestion. On the basis of the amino acid composition and the minimum molecular weight determined from the coenzyme content, the total number of peptides to be expected after tryptic digestion is 46. Since the enzyme is known to contain 2 molecules of bound pyridoxal phosphate per molecule of protein (2, 13), it seems logical that the enzyme consists of two identical (or very similar) subunits, each weighing approximately 47,000. The difference between the number of tryptic peptides predicted for this value (*i.e.* 46) and the number of peptides found experimentally (*i.e.* 38) could be due to incomplete separation, to the loss of some of the peptides, or to the occurrence of contiguous basic amino acid residues in the protein. An alternative explanation is that the value of the assumed molecular weight is too high.

The conclusion that the enzyme consists of two identical subunits agrees with the view of Polyanovskii and Vorotnitskaya (49); these authors detected up to 50 peptides in the tryptic hydrolysate of cytoplasmic aspartate aminotransferase and assigned to the subunit a molecular weight of about 55,000 (110,000 for the dimeric holoenzyme). Recent studies (50, 51) ascribe to the dimeric enzyme a molecular weight of 90,000 or less, which is in fair agreement with that predicted from the coenzyme content (47,000 × 2 = 94,000).

The similarity in the chemical composition of the subforms of cytoplasmic aspartate aminotransferase was confirmed by the immunological studies. It is interesting that Wada and Morino (6) and Nisselbaum and Bodansky (9) could show a marked immunological difference between the cytoplasmic and mitochondrial enzymes.

The behavior of cytoplasmic transaminase in the ultracentrifuge has been investigated in several laboratories (2, 6, 46, 50–53). Most of the data for the sedimentation coefficient agree on a value for $s_{20,w}$ of 5.5. These values were obtained with preparations that contained various subforms or substantial amounts of inactive material. Some of the studies (2) showed a remarkable lack of concentration dependence of the sedimentation coefficient. Present data show that the main subforms of cytoplasmic transaminase behave similarly in the ultracentrifuge; the sedimentation coefficient ($s_{20,w}$) calculated by extrapolation to infinite dilution is 5.7 S (Fig. 10). The finding that at a protein concentration of less than 0.2 g/100 ml the experimentally determined sedimentation coefficients of subform α fall below the straight line connecting the values obtained at higher concentrations might suggest that at low concentration the α subform begins to dissociate into subunits. The concentration at which this effect is observed, however, is close to the limits of applicability of the schlieren optics used in the present study, so that the departure from linearity of the experimental points is barely outside the limits of experimental error. On the other hand, a dissociation of aspartate aminotransferase into subunits has been postulated (54) on the basis of fluorescence polarization studies.

Information about the conformation of the subforms is provided by optical rotatory dispersion studies. In the far ultraviolet portion of the curve, which is thought to reflect essentially the over-all protein conformation (55, 56), no large difference was found except for the fact that some preparations of Fraction I gave slightly less negative values around 232 mμ. The difference, however, was of the same order as the experimental error. The negative trough at 232 mμ of all fractions was somewhat shallower after the material had been subjected to the treatment for dissociating the "active" coenzyme. According to current views (55, 56), this could indicate that a slight loss of helical content has taken place (26).

In the near ultraviolet and visible portion, the optical rotatory dispersion of both Fractions IV (α subform) and II (β subform) presents very evident anomalies centered around the absorption peaks of the bound coenzyme. The amplitude of these anomalies is slightly greater than that previously reported for a mixture of the subforms (26, 57). This can be easily explained since the mixture contained material with an absorption peak at 340 mμ. As shown in Fig. 11, the optical rotatory dispersion curve of the material from Fraction I, which had been treated to remove the "active" coenzyme and thus contained only "inactive" coenzyme absorbing at 340 mμ, is plain. This suggests that in the "inactive" material the coenzyme is *not* asymmetrically bound as in the active enzyme molecules. However, more sensitive methods, *e.g.* the determination of circular dichroism (58), might reveal the presence of a Cotton effect at 340 mμ which is too small to be detected by optical rotatory dispersion studies.

Before Fraction I has been treated to detach the "active" coenzyme, it presents a shallow anomaly centered around 430 mμ at acidic pH or at 360 mμ at alkaline pH. This anomaly may be attributable to the small amount of active enzyme present in Fraction I.

The effect on cytoplasmic aspartate aminotransferase of exposure to concentrated urea has been studied by several investigators (37, 38); in the presence of 8 M urea the activity of the enzyme undergoes a rapid and reversible fall to 25% of the original and then decays more slowly until it practically vanishes (37). The rate of this activity loss cannot be described by a simple equation (37). Changes in the spectral properties and in optical rotation suggest that an extensive conformational modification occurs in parallel with the slow activity loss (37). The quantum yield of the coenzyme fluorescence markedly increases after incubation in concentrated urea (38, 54).

An investigation of the behavior of the transaminase subforms in urea was interesting from two points of view. On the one hand, it seemed possible that the complicated kinetics for the urea-induced structural changes observed with a mixture of the subforms could, at least in part, be due to a different behavior of the various subforms; on the other hand, it was important to establish whether the three main subforms remained distinguishable after their structure had been perturbed by concentrated urea. The rate of the slow conformational changes consequent to exposure to concentrated urea is faster for the α subform than for the β subform. Since the α subform also reacts with pyridoxal phosphate much faster than the β subform and, possibly, begins to dissociate into subunits at higher concentration, it seems reasonable to think that the structure of the α subform is less rigid or less compact than that of the β subform, even though such differences do not appear in the optical rotatory dispersion curve.

Whatever conformational differences may exist among the subforms, it is interesting that, even after the conformation has been deeply perturbed by exposure to concentrated urea for many

hours, the subforms still preserve their electrophoretic individuality. The whole of the chemical and physicochemical evidence suggests that the differences in the primary structure of the subforms, if any, must be so small as to escape detection by amino acid analysis and peptide mapping of the tryptic digests, and that a difference in the secondary and tertiary structure probably occurs in that part of the enzyme molecule which preserves some ordered structure even in concentrated urea (26).

The presence of several active fractions in an enzyme preparation can be attributed either to modifications of some of the enzyme molecules during the purification procedure (as is the case with hexokinase) (59) or to the occurrence of biologically determined subforms (as is the case with several dehydrogenases) (1).

The possibility that the aspartate aminotransferase subforms may be produced by artificial modifications must be considered. Artificial modifications definitely do occur when the enzyme is exposed to maleate, which has been extensively used as a protective buffer in the preparation of the enzyme. As shown in Fig. 12, exposure of the various fractions to maleate caused the appearance of many new electrophoretic bands. Since maleate can bind to —SH groups of the enzyme to form stable thioether derivatives (40), the molecules of each chromatographic fractions might react with a varying number of maleate molecules, generating new families of subforms. The figure of approximately one out of six —SH groups etherified by maleate determined in a mixture of cytoplasmic aspartate aminotransferase (40) must therefore represent an average.

None of the treatments used during the purification procedure described in this paper, if applied separately to any of the isolated fractions, modified its electrophoretic individuality. Experiments carried out so as to reduce to a minimum the probability of producing artificial modifications of the enzyme showed that the multiple forms are present in a cold water homogenate from a single heart analyzed soon after the death of the animal. If the subforms are produced by an artifact, it must occur very early in the preparation, either in the cell soon after the death of the animal or during homogenization in the cold. The fact that the subforms are present in material obtained from a single heart is also significant because it excludes the possibility that the various subforms originate from the hearts of different individuals.

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