Molecular Properties of the Multiple Aspartate Aminotransferases Purified from Rat Brain*

Steve C. Magee† and Allen T. Phillips‡

ABSTRACT: The cytoplasmic and mitochondrial aspartate aminotransferases from rat brain have been purified to a state of homogeneity as determined by immunological, ultracentrifugal, and electrophoretic criteria. The two isoenzymes each have molecular weights of approximately 80,000 and $s^0_{20,w}$ values of 5.4 S. The amino acid compositions of the two principal enzyme forms are markedly different, however, in keeping with their opposite electrophoretic behavior at neutral pH. Antibodies prepared against either purified enzyme fail to cross-react with the other isoenzyme when tested in pure form or in crude homogenates. The cytoplasmic aspartate aminotransferase has a 10-fold lower K_m for α-ketoglutarate than does the mitochondrial enzyme, whereas the latter enzyme exhibits a 10-fold reduction in K_m for aspartate over the cytoplasmic form. Other Michaelis constants are quite similar. Oxaloacetate and glyceraldehyde 3-phosphate are inhibitors of glutamate transamination in both enzymes, whereas inorganic phosphate is inhibitory only toward the mitochondrial isoenzyme. The mitochondrial enzyme was separated by CM-Sephadex chromatography into three distinct fractions. The predominant mitochondrial aminotransferases (M-II and M-III) have absorption maxima near 350 nm at pH 7.5, whereas the less prominent M-I fraction absorbs maximally at 330 nm. The amino acid compositions and Michaelis constants of M-II and M-III are essentially identical but the enzymes differ in observed specific activities. The relative amounts of the three variants are altered upon storage of purified preparations, implying a time-dependent conversion of forms M-II and M-III into the less active M-I. Cytoplasmic aspartate aminotransferase in the freshly purified state revealed no subforms but two electrophoretically distinct forms are present in aged preparations of purified enzyme and may also occur in crude extracts of rat brain cytoplasm.

The presence of two distinct isoenzymes of AAT[1] (EC 2.6.1.1) in mammalian liver and heart has been well documented (Fleisher *et al.*, 1960; Wada and Morino, 1964; Boyd, 1966). One of these, the cationic AAT, is localized in the mitochondrial fraction of these tissues, whereas the other, an anionic form, is restricted to the cytosol fraction. Aspartate aminotransferases isolated from these two cell regions have been shown to possess different K_m values and pH maxima (Fleisher *et al.*, 1960; Boyd, 1961; Nisselbaum and Bodansky, 1966; Boyd, 1966), tryptic digest peptide maps, and amino acid compositions (Martinez-Carrion and Tiemeier, 1967). Martinez-Carrion *et al.* (1965) have also reported that several subforms are associated with each AAT isoenzyme from pig heart and these are apparently present in the tissue *in vivo*. The subforms are distinguishable by their specific activities and by the spectral characteristics of pyridoxal phosphate bound to each, but they appear similar in substrate affinities.

Although most of the information to date regarding the properties and biological functions of AAT has been derived from studies on liver and heart, preliminary studies with partially purified AAT from brain have been carried out (Bonavita, 1959; Pattabhiraman and Bachhawat, 1959). The present investigation was undertaken to determine more fully some of the properties of brain AAT so that a better understanding of its contribution to glutamate metabolism in brain might be possible.

* From the Department of Biochemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received February 24, 1971.*

† Present address: Department of Biochemistry, Oklahoma State University, Stillwater Okla.

‡ To whom correspondence should be addressed.

[1] Abbreviations used are: AAT, aspartate aminotransferase; s-AAT, cytoplasmic (or soluble) aspartate aminotransferase m-AAT, mitochondrial aspartate aminotransferase.

Materials and Methods

Special Products. The following materials were obtained commercially: potassium aspartate, oxaloacetic acid, potassium α-ketoglutarate, ribonuclease (type 1-A), DL-glyceraldehyde 3-phosphate (diethyl acetal), and malate dehydrogenase (type 410-13) from Sigma Chemical Co., St. Louis, Mo.; monosodium L-glutamate from General Biochemicals, Chagrin Falls, Ohio; pyridoxal 5-phosphate from Mann Research Corp., New York, N. Y.; Sephadex gels from Pharmacia, Uppsala, Sweden; bovine serum albumin from Pentex Inc., Kankakee, Ill.; NADH from P-L Biochemicals, Milwaukee, Wis.; coomassie blue and Ionagar No. 2 for immunodiffusion from Colab Laboratories, Chicago Heights, Ill.; 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride from Pierce Chemical Co., Rockford, Ill.; and Freund's complete adjuvant from Difco Laboratories, Detroit, Mich.

Enzyme Assays. Routine AAT assays were performed by a modification of the method of Amador *et al.* (1967). In a final volume of 0.29 ml were contained 7.6 μg of sulfate-free malate dehydrogenase, 20 μmoles of potassium phosphate, 36 μmoles of potassium aspartate, 0.144 μmole of NADH, and 2 μmoles of potassium α-ketoglutarate, in addition to an appropriate amount (0.5–10.0 munits) of AAT; the final pH was 7.4 and the temperature was 30°. A unit of enzyme is defined as the amount of AAT which results in the production of 1 μmole of oxaloacetate/min under the conditions of the assay described. Absorbance changes were followed at 340 nm in a Gilford Model 2000 spectrophotometer.

For the determination of kinetic constants in the direction of aspartate formation, the change in absorbance at 280 nm due to oxaloacetate disappearance was measured in a cuvet (0.25-ml final volume) containing 15 μmoles of monosodium glutamate, 0.42 μmole of oxaloacetate, and 17.3 μmoles of potassium phosphate, with a final pH of 7.4. The molar ab-

sorptivity of oxaloacetate is 550 at 280 nm (Nisonoff and Barnes, 1952). In some assays oxaloacetate concentration was varied from that stated here.

For maximum precision of AAT assays, phosphate concentration was kept constant at 69 mM and sulfate concentration was less that 2 mM. Variations in anion nature and concentration have been reported by Boyde (1968) to affect drastically the m-AAT and s-AAT from mammalian liver.

Ultracentrifugation. Sedimentation velocity runs were performed in a Spinco Model E ultracentrifuge at either 10 or 20° with protein which previously had been dialyzed against 0.05 M potassium phosphate (pH 7.4). Protein concentrations ranged from 0.075 to 0.55%. A photoelectric scanner was employed for the lower protein concentrations, while conventional schlieren optics were used at higher concentrations. Rotor speed was 60,000 rpm.

Sedimentation equilibrium ultracentrifugation was conducted at 20° in the Spinco Model E ultracentrifuge with the photoelectric scanner operated at 280 nm for protein concentrations between 0.1 and 0.5 mg per ml and at 303 nm for protein concentrations between 0.5 and 1.5 mg per ml. The solvent was 0.05 M potassium phosphate (pH 7.4). Enzyme solution (usually 0.15 ml) was layered onto FC-43 fluorocarbon oil in one sector of the Al-filled Epon centerpiece, and 0.18 ml of solvent was placed in the other sector. The centrifuge was operated at 18,000 rpm for 45 min, then maintained at 11,000 rpm for at least 17 hr. Molecular weights and partial specific volumes were determined by the method of Edelstein and Schachman (1967) in water and 98% D_2O.

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed at pH 9.0, as described by Davis (1965), and at pH 7.0, as suggested by Williams and Reisfield (1964). Coomassie blue (0.1% in 10% trichloroacetic acid) was used as a general staining agent for protein and aspartate aminotransferases were specifically located by the method of Dekker and Rau (1963) with 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride as the staining reagent.

Paper electrophoresis was carried out in a Durrum-type chamber on strips (3 × 30 cm) presoaked in 0.07 M potassium phosphate (pH 6.5). Running time was 25 hr at 5° with the current maintained at 1 mA/strip.

Cellulose acetate electrophoresis was conducted on 1 × 6.75 in. Sepraphore III strips (Gelman Instrument Co., Ann Arbor, Mich.) in Gelman high-resolution buffer. Voltage was maintained at 300 V for 1 hr, at a temperature of 23°.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Amino Acid Analysis. All determinations were performed on a Technicon automatic amino acid analyzer. Enzyme was dialyzed against 1 l. of 20 mM NaCl for 3 hr, 1 l. of 1 mM NaCl for 14 hr, and distilled water for 14 hr. The salt-free protein was lyophilized and then hydrolyzed in an evacuated sealed glass tube for 24 hr in 6 N HCl at 110°. Duplicate samples of each hydrolysate were analyzed.

Immunological Assays. Crystalline s-AAT and m-AAT (fraction M-III) were individually dissolved in a small amount of water and dialyzed 14 hr against 350 ml of 1% NaCl. Freund's complete adjuvant (1 ml) was mixed with 1 ml (3.1 mg) of the dialyzed enzyme and 1 ml was injected subcutaneously into the abdominal region of adult white rabbits. Twenty-eight days later a similar injection of 1 ml was made into each animal. Eighteen days after the second injection, blood samples were taken from each animal by ear puncture. The individual blood samples were kept in ice for 6 hr, then centrifuged to obtain the serum. Serum samples were stored frozen until needed. The antisera obtained were used directly for immunological analysis by the Ouchterlony method (Stollar and Levine, 1963).

Results

Purification of Rat Brain Cytoplasmic Aspartate Aminotransferase. All steps were carried out at 0–5° unless specified otherwise. Ammonium sulfate concentrations are expressed as per cent of saturation at 0°.

STEP 1. One-hundred eighty-five Sprague–Dawley descendants weighing 200–350 g were decapitated, the brains quickly removed, and washed with 0.25 M sucrose–0.1 mM EDTA (pH 7.5). Portions containing approximately 4 g of brain were homogenized in 25 ml of the wash solution in a size B Teflon tissue grinder (Arthur H. Thomas, Philadelphia, Pa.) for 1 min. The resulting combined homogenates were centrifuged at 700*g* for 10 min. The precipitate was resuspended in 1.5 l. of the sucrose–EDTA solution and centrifuged for 10 min at 1800*g*. The last step was repeated and the pooled supernatants were centrifuged at 40,000*g* for 45 min. The precipitate of mitochondria and other cell particulates was stored at −15° for later preparation of mitochondrial AAT.

STEP 2. The 40,000*g* supernatant from step 1 was made 0.2 M in potassium phosphate by the addition of 2 M potassium phosphate (pH 7.5). Solid $(NH_4)_2SO_4$ was added slowly to the solution to 53% saturation (312 g/l.) and the resulting mixture stirred for 30 min. The precipitate which formed was removed by centrifugation at 10,000*g* for 20 min. The supernatant was brought to 85% saturation by the addition of 250 g of $(NH_4)_2SO_4$/l. of solution, and stirred for 1 hr. The precipitate was recovered by centrifugation as before. The final precipitate was taken up in 5 mM potassium phosphate–2.5 mM EDTA buffer (pH 7.4), such that the final volume was approximately 2.5% of the original homogenate volume in step 1.

STEP 3. Crystalline potassium α-ketoglutarate and ribonuclease were added to final concentrations of 2 mM and 0.5 mg/ml, respectively. The protein solution was allowed to warm to 25° with occasional stirring. The solution was then made 0.1 M with respect to succinate (Turano *et al.*, 1964) and phosphate by the addition of 1 M solutions of the potassium salts (pH 7.4). Portions of 20 ml were placed in 50-ml polypropylene centrifuge tubes and were heated to 70° while held in a boiling-water bath. The samples were maintained at 70° for 10 min, then cooled quickly to 40° by placing the centrifuge tubes in an ice bath. The tubes were removed from the ice bath and allowed to stand at room temperature for 1 hr. The precipitate was removed by centrifugation. In most purifications, at this point the supernatant was frozen in a Dry Ice–acetone bath and maintained at −15° until fractionation could be continued.

STEP 4. The supernatant fraction from step 3 was thawed and $(NH_4)_2SO_4$ was added (600 g/l., 90% saturation) with stirring. After standing at 0–5° overnight, the precipitate was collected by centrifugation at 12,000*g* for 15 min. The precipitate was taken up in 10 ml of 5 mM potassium phosphate (pH 7.5), dialyzed 4 hr against 1 l. of 10 mM potassium phosphate buffer (pH 7.5), which was 0.1 mM with respect to magnesium acetate and 0.01% in 2-mercaptoethanol and further dialyzed for 2 hr against 2 l. of 5 mM potassium phosphate–2 mM α-ketoglutarate (pH 7). The dialyzed enzyme solution was made 40% by volume in acetone through the addition of acetone precooled to −10°. The mixture was stirred for 10 min while maintaining it at −10° in a salt–ice bath. A precipitate was

TABLE I: Purification of Rat Brain Cytoplasmic Aspartate Aminotransferase.

Step	Vol (ml)	Total Act.[a] (Units)	Total Protein (mg)	Sp Act. (Units/mg)	Recov (%)
1. 40,000*g* supernatant	6200	7600	9750	0.78	100
2. Ammonium sulfate fractionation (53–85% fraction)	151	5300	1300	4.0	70
3. Heat treatment[b]	152	5210	345	15.1	69
4. Acetone fractionation (40–60%)	23	5150	316	16.3	68
5. Sephadex G-200	90	3480	69.7	50.0	46
6. DEAE-Sephadex	61	3190	13.4	238	42
7. Crystallization (57–63% fraction)	3	1600	6.7	239	21

[a] The units are given as micromoles of oxaloacetate produced per minute. [b] In the absence of α-ketoglutarate, only 77% of the step 2 activity is recovered.

removed by centrifugation at 12,000*g* for 5 min at −10°. The supernatant was then adjusted to 60% acetone and maintained at −15° for 1 hr. The latter precipitate was collected by centrifugation at −15° and dissolved in 10 ml of 0.1 M potassium phosphate (pH 7.4).

STEP 5. The enzyme solution was applied to a 3.6 × 95 cm Sephadex G-200 column which was equilibrated and eluted with 10 mM potassium phosphate (pH 7.4). Fractions of 15 ml were collected at a flow rate of 20 ml/hr.

STEP 6. The fractions containing the highest specific activity were pooled, diluted with two volumes of distilled water, and applied to a DEAE-Sephadex (A-50) column (1.2 × 35 cm), equilibrated with 5 mM potassium phosphate (pH 7.5). The column was washed with 50 ml of the same buffer and then eluted with a linear gradient (500 ml) from 5 to 50 mM phosphate. AAT elution from the column began at 16 mM and was complete by 25 mM. The enzyme eluted as a single symmetrical peak.

The pooled active fractions were diluted with three volumes of distilled water and applied to a second DEAE-Sephadex column (0.5 × 5 cm) equilibrated with 5 mM potassium phosphate (pH 7.8). The enzyme was eluted as a concentrated band with 0.1 M potassium phosphate (pH 7.4).

STEP 7. Crystallization was carried out by the back-extraction procedure of Jakoby (1965). The enzyme was precipitated by adding $(NH_4)_2SO_4$ to 90% saturation. Solutions of $(NH_4)_2SO_4$ from 75 to 54% saturation at 3% intervals were prepared by diluting an appropriate volume of saturated $(NH_4)_2SO_4$ containing 50 mM potassium phosphate–2 mM α-ketoglutarate (pH 8) with a similar solution lacking $(NH_4)_2SO_4$. The precipitated enzyme was first suspended in the 75% saturated solution and centrifuged at 7000*g* for 5 min at 5°. The supernatant fluid was decanted and allowed to stand at room temperature. The residue was then resuspended in 0.5 ml of the next lower salt concentration, and the process repeated sequentially to 54% saturation. Flat crystals appeared within 24 hr in the solutions from 63 to 57% saturation; however, within 5 days the crystals had reverted to an amorphous form. This transition is similar to that reported by Wada and Morino (1964) for bovine heart AAT. A summary of the purification scheme is given in Table I.

Purification of Rat Brain Mitochondrial Aspartate Aminotransferase. All centrifugations were carried out at 0–5° and 27,000*g* for 10 min.

STEP 1. The 40,000*g* precipitate (240 ml) obtained from step 1 of the purification of the cytoplasmic enzyme was mixed with 280 ml of 0.1 M potassium phosphate–10 mM α-ketoglutarate buffer (pH 6.6). The mixture was stirred until smooth, then 0.25 volume of *n*-butyl alcohol cooled to 0° was added and vigorous stirring continued for 10 min. The mixture was centrifuged and the aqueous phase, including any precipitate, collected. The precipitate was resuspended in 200 ml of the same buffer and homogenized for 1 min in a Waring Blendor at low speed. Again a 0.25 volume of cold *n*-butyl alcohol was added and the suspension stirred for 10 min. The aqueous phase was obtained by centrifugation as above. The precipitate was resuspended by blending as before in 160 ml of potassium phosphate–α-ketoglutarate buffer and 0.1 volume of cold *n*-butyl alcohol was added and stirred for 10 min. The aqueous phase was obtained by centrifugation and pooled with the others. The combined aqueous phases were allowed to stand in an ice bath for 2 hr and the organic layer was removed by aspiration. The aqueous layer was dialyzed against two changes, each 6 l., of 0.05 M potassium phosphate–1 mM α-ketoglutarate (pH 7.4) for 12 hr each. The tubing contents were centrifuged and the supernatant saved.

STEP 2. $(NH_4)_2SO_4$ (348 g/l., 58% saturation) was added to the supernatant from step 1 and the solution was stirred for 1 hr. After centrifugation, $(NH_4)_2SO_4$ (187 g/l., 83% saturation) was added and the solution stirred for 1 hr. The precipitate was collected by centrifugation and suspended in 10 ml of water. The suspension was dialyzed against 250 ml of 0.01 M potassium phosphate–1 mM α-ketoglutarate (pH 7.0) for 15 hr.

STEP 3. The tubing contents were centrifuged to remove any insoluble material and the supernatant was applied to a 3.6 × 95 cm Sephadex G-200 column equilibrated with 0.02 M potassium phosphate buffer (pH 7.0). Elution was with this same buffer and the most active fractions were pooled and dialyzed against 4 l. of 0.015 M potassium phosphate (pH 6.4) for 12 hr.

STEP 4. The dialyzed enzyme from step 3 was applied to a 1.5 × 40 cm CM-Sephadex column equilibrated with 0.015 M potassium phosphate (pH 6.4). The column was eluted with a 1500-ml linear gradient of potassium phosphate (pH 6.4, 0.02–0.10 M). Fractions of 10 ml were collected with a flow rate of 40 ml/hr. Active fractions were pooled into three groups as indicated in Figure 1.

Each of the pooled samples was diluted with 4 volumes of distilled water and applied to separate 0.7 × 10 cm CM-Sephadex columns equilibrated with 0.01 M potassium phosphate (pH 6.4). Enzyme was eluted as a concentrated band by

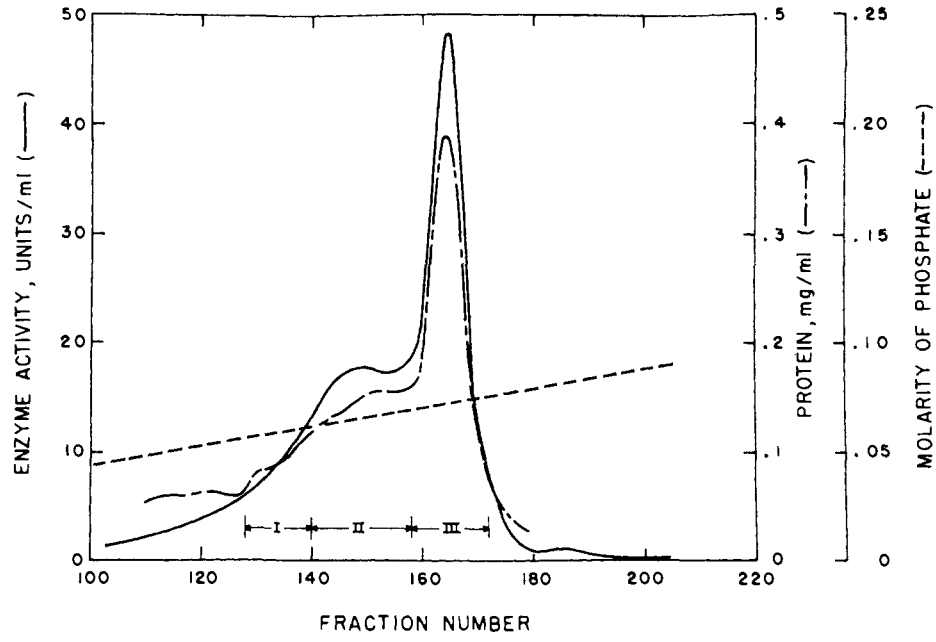

FIGURE 1: Elution pattern of m-AAT from CM-Sephadex. Protein (300 mg, specific activity 27.2) from step 3 was applied to a column of CM-Sephadex and eluted in a gradient of 0.02 M potassium phosphate (pH 6.4) to 0.10 M in 10-ml fractions.

0.1 M potassium phosphate (pH 6.4). No purification was gained by this procedure but a 10- to 20-fold concentration could rapidly be effected with no loss in activity.

STEP 5. The three concentrated fractions from step 4 were further concentrated individually by precipitation with $(NH_4)_2SO_4$ (final concentration, 90%), collected by centrifugation, and dissolved in 0.5 ml of 0.1 M potassium phosphate–0.02 M α-ketoglutarate (pH 7.0). Ammonium sulfate was added slowly with stirring to fractions II and III until a precipitate just began to form. The small amount of precipitate was removed by centrifugation and the yellow supernatant was stored at 0° in an ice bath. Needle-like crystals began to appear in each within 1 week and crystallization continued for several weeks. No attempt was made to crystallize fraction M-I due to insufficient material. A summary of this purification is given in Table II.

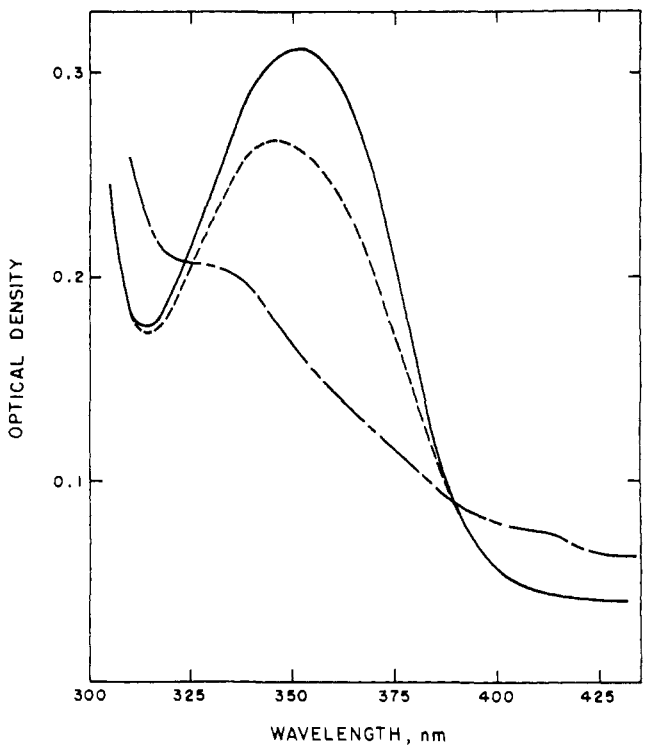

FIGURE 2: Absorption spectra of M-AAT fractions I, II, and III. Protein concentrations were 1.25 mg/ml for M-I (—·—·—), 2.44 mg/ml for M-II(- - -), and 2.50 mg/ml for M-III (———) in 0.05 M potassium phosphate (pH 7.5).

The purifications of the two aspartate aminotransferases have been conducted a number of times. All operations were generally quite reproducible with the exception of the acetone fractionation step employed in isolating the cytoplasmic aspartate aminotransferase. In this case, some variability was experienced in the extent of purification achieved (ranging from 1- to 10-fold increase in specific activity) and in the recovery of activity. Complete success at this step may not be essential since the DEAE-Sephadex column generally gave rise to an enzyme solution which exhibited a single band upon disc electrophoresis regardless of the degree of purification obtained in the acetone step. Crystallizability is improved when the acetone step is included.

Preliminary work with the mitochondrial enzyme revealed that insufficient removal of the *n*-butyl alcohol by dialysis in step 1 prevented complete precipitation of the enzyme by $(NH_4)_2SO_4$. The m-AAT was not sufficiently stable at 60° to employ a heat treatment in its purification.

Gradients for the CM-Sephadex column which were less steep than that used in the representative purification for m-AAT gave more distinct separation of the major peak (fraction III) from the remaining fractions. It is recommended that a linear gradient generated from 1 l. of 0.02 M potassium phosphate mixed with 1 l. of 0.08 M potassium phosphate (pH 6.4) be used to elute the CM-Sephadex column if optimum separation of fractions II and III is desired.

Physical Properties of the Aspartate Aminotransferases. Crystals obtained from the cytoplasmic and mitochondrial

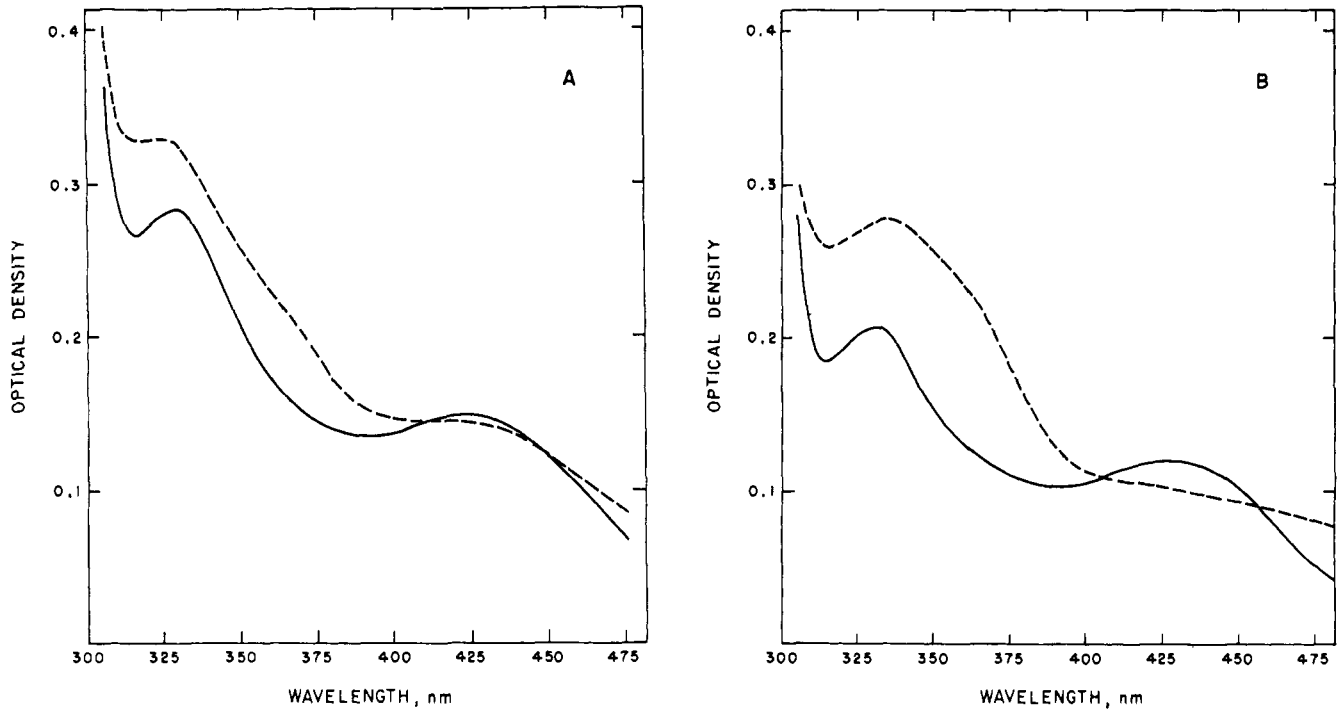

FIGURE 3: Absorption spectra of aged m-AAT. Each protein sample was dialyzed against 0.05 M potassium phosphate buffer overnight and then centrifuged. (A) Fraction M-II; (———) pH 5.0, (- - -) pH 7.5, (B) Fraction M-III; (———) pH 5.0, (- - -) pH 7.5. All protein concentrations were 2.5 mg/ml.

TABLE II: Purification of Rat Brain Mitochondrial Aspartate Aminotransferase.

Step	Vol (ml)	Total Act.[a] (Units)	Total Protein (mg)	Sp Act. (Units/mg)	Recov (%)
1. Dialyzed butanol extract	885	12,100	1870	6.4	100
2. Ammonium sulfate (58–83%)	20.5	11,000	625	18.0	91
3. Sephadex G-200 chromatography	140	8,170	300	27.2	68
4. CM-Sephadex chromatography					
Fraction I	120	1,020	9.8	104	8
Fraction II	191	3,030	23.5	111	21
Fraction III	189	4,320	33.6	125	36
5. Crystallization					
Fraction II	1.15	1,170	10.1	116	10
Fraction III	1.40	2,170	13.0	167	18

[a] Micromoles of oxaloacetate produced per minute.

aspartate aminotransferase were of different types; the cytoplasmic enzyme crystallized as platelets while the mitochondrial enzymes (fractions II and III) formed needles. Whether such variation reflects different tendencies toward a particular crystal form or is caused by the nonidentical procedures used in crystallization cannot be decided at this time.

The spectra of these enzymes are largely similar to those recorded for s-AAT and m-AAT from other sources (Wada and Morino, 1964; Martinez-Carrion and Tiemeier, 1967; Michuda and Martinez-Carrion, 1969). In fractions M-II and M-III, maximum absorption (above the 280-nm region) occurred at 350 ± 5 nm for pH 7.5 (Figure 2); at pH 5.0, an increase in absorption at 425 and 330 nm was noted, with a concomitant decrease in the 350-nm peak. In contrast, fraction M-I at pH 7.5 exhibited more absorption at 330 nm than at 350 nm (Figure 2).

Upon the aging of fractions M-II and M-III for 6 months at −15° the spectral peaks at 350 nm were lost in favor of new maxima at 330 nm (Figure 3). These 330-nm peaks were seen at both pH 5.0 and 7.5. Aged fraction III did, however, maintain a detectable shoulder at 355 nm at pH 7.5.

These findings are consistent with the interpretation that the 425- and 350-nm peaks are due to a pH-sensitive phosphopyridoxal–aldimine enzyme complex while the 330-nm component represents a catalytically unfavorable form, perhaps resulting from a nonspecific binding of pyridoxal phosphate to groups other than those normally participating in the phosphopyridoxal–aldimine enzyme (Michuda and Marti-

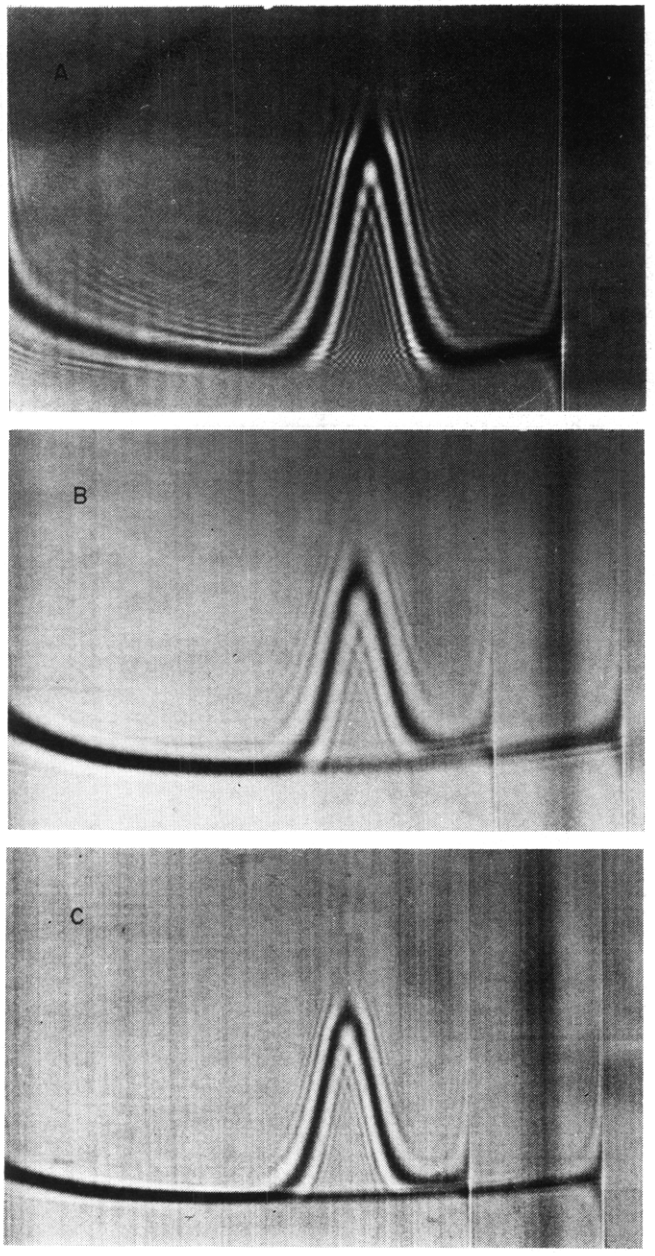

FIGURE 4: Sedimentation velocity patterns for AAT. (A) s-AAT; 5.0 mg/ml in 0.05 M potassium phosphate (pH 7.5); bar angle, 60° (B) m-AAT fraction II; 5.5 mg/ml in 0.05 M potassium phosphate, pH 7.5; bar angle, 55° (C) m-AAT fraction III; 5.5 mg/ml in 0.05 M potassium phosphate, pH 7.5; bar angle, 65° photographs were made approximately 32 min after reaching 60,000 rpm. Sedimentation is shown from right to left.

nez-Carrion, 1969). Aging of enzyme samples promotes an increase in the pH-insensitive 330-nm absorption in the cases of M-II and M-III enzyme, bringing their absorption spectra more nearly like that of fraction M-I (compare Figures 2 and 3).

Sedimentation coefficients were determined in 0.05 M potassium phosphate (pH 7.5) and corrected to $s_{20,w}$ as described by Schachman (1957). Velocity experiments indicated only a single component in each of the protein samples (Figure 4). There was a slight negative slope in the plot of $s_{20,w}$ *vs.* concentration for s-AAT, and the value of 5.40 S was determined for $s^0_{20,w}$ by extrapolation to zero concentration. The analogous $s^0_{20,w}$ values for mitochondrial aspartate aminotransferase fractions M-II and M-III were 5.32 and 5.40 S, respectively.

TABLE III: Amino Acid Composition of the Aspartate Aminotransferases.

Amino Acid	Residues/80,000 g of Enzyme[a]		
	Cytoplasmic Enzyme	Mitochondrial Fraction II	Mitochondrial Fraction III
Aspartic acid	77	66	64
Threonine	36	34	33
Serine	41	44	46
Glutamic acid	66	74	73
Proline	43	33	32
Glycine	52	67	64
Alanine	55	62	61
Half-cystine	6	11	12
Valine	47	52	51
Methionine	12	17	18
Isoleucine	26	34	35
Leucine	63	59	58
Tyrosine	20	21	22
Phenylalanine	43	31	31
Ammonia	89	132	116
Lysine	36	50	50
Histidine	13	17	16
Arginine	38	33	37
Tryptophan	17	11	11

[a] Rounded to nearest whole number.

Sedimentation equilibrium ultracentrifugation with protein concentration between 0.01 and 0.15% gave consistent molecular weights of 79,000 ± 3000 for s-AAT. The mitochondrial fractions M-II and M-III had apparent molecular weights of 81,000 ± 4000 each. Approximate partial specific volumes for each were calculated on the basis of amino acid composition (Cohn and Edsall, 1943) distributing NH_3 equally between asparagine and glutamine and did not differ appreciably from values obtained by centrifugation in H_2O and D_2O (Edelstein and Schachman, 1967). The values obtained by the latter method were 0.734, 0.736, and 0.735 ml per g for s-AAT and m-AAT fractions M-II and M-III, respectively.

Electrophoretic Properties. Crystalline s-AAT was homogeneous on disc electrophoresis at pH 7 and 9. However, preparations of the crystalline s-AAT which had been stored for several months revealed an additional protein band upon electrophoresis. This new component migrated faster than the "parent" protein and demonstrated enzymatic activity, although its specific activity could not be accurately estimated. The component was not eliminated by 48-hr incubation of the aged enzyme with 1 mM α-ketoglutarate, 5 mM aspartate, 0.2 mM pyridoxal phosphate, or 50 mM mercaptoethanol.

Paper electrophoresis of freshly prepared M-III fractions usually indicated there was approximately 10% contamination resulting from the protein of fraction M-II. A comparison of enzyme-specific stains with general protein stains indicated that the samples were otherwise free of contaminating proteins. Cellulose acetate electrophoresis at pH 8.8 in Tris–barbital buffer (Gelman high-resolution buffer), revealed that both components migrated as cations under these conditions. Isolated fraction M-II from the CM-Sephadex column con-

TABLE IV: Apparent Michaelis Constants for the Aspartate Aminotransferases.

AAT Isoenzyme	Michaelis Constants (mM)			
	Asp	α-KG	GA[a]	OAA
Cytoplasmic	6.7	0.15	5.0	0.11
Mitochondrial				
Fraction I	0.78	1.7	7.8	
Fraction II	0.61	1.5	6.5	
Fraction III	0.50	1.3	3.5	0.10

[a] The values for glutamate were obtained at an oxaloacetate concentration of 1.68 mM.

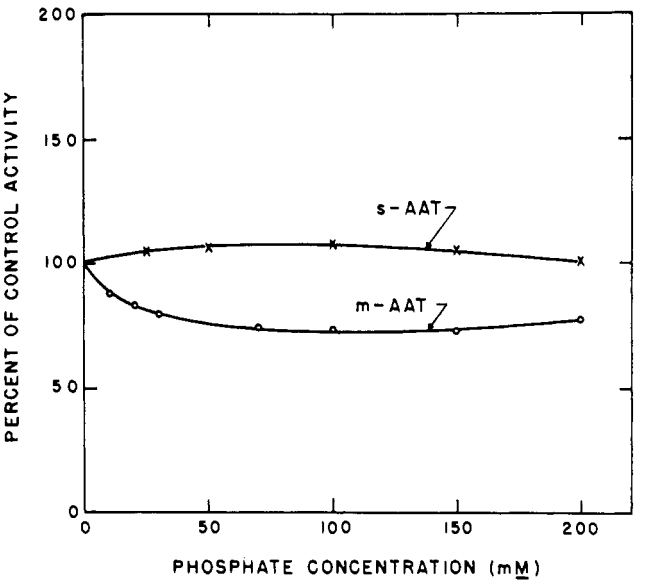

FIGURE 5: Effect of potassium phosphate on AAT activity. Samples of purified s-AAT or m-AAT (predominantly fraction III) were dialyzed overnight against 2 mM sodium barbital buffer (pH 7.4) then diluted 1:50 in a solution of bovine serum albumin (2 mg/ml) adjusted to pH 7.4 with dilute NaOH. A total of 3 munits was then placed into each assay mixture containing all the normal components at pH 7.4 except phosphate concentration was varied as indicated. Initial rates of oxaloacetate formation were then taken for comparison since linearity of product formation was observed only in the early phases if the solutions were relatively unbuffered.

tained as much as 25% contamination from fractions M-I and M-III.

Amino Acid Analyses. Results of the amino acid analyses are presented in Table III. The values stated are averages of duplicate determinations and are uncorrected for hydrolytic losses. Tryptophan was determined by averaging the results obtained from two spectrophotometric methods (Goodwin and Morton, 1946; Bencze and Schmidt, 1957). The method of Goodwin and Morton gave a ratio of 1.9 for tyrosine to tryptophan in m-AAT whereas that by the Bencze and Schmidt procedure was 2.0. Similar agreement between methods was found in the analyses performed on s-AAT.

Brain m-AAT fractions have different amino acid compositions from brain s-AAT. The m-AAT forms contain more lysine than does s-AAT and it is likely that most of the acidic amino acids of m-AAT are present as amide forms. Thus the compositions are in general agreement with the observations that the cytoplasmic and mitochondrial enzymes migrate in opposite directions during electrophoresis at neutral pH. The presence of more aromatic residues in s-AAT than in m-AAT accounts for the higher extinction coefficient at 280 nm ($E^{1\%}_{280}$ 13.5 *vs.* 10.8 at pH 7.5).

Fractions M-II and M-III of m-AAT have amino acid compositions which are identical within the limits of the analytical method used, except for the determination of ammonia. This may indicate that the differing electrophoretic and chromatographic mobilities of these subforms are due in part to variations in amide content.

Immunological Reactivity. Antisera prepared against the purified s-AAT and m-AAT fraction III were found to give only a single precipitin band when tested by gel diffusion against the corresponding antigen or against crude homogenates. No inhibition of s-AAT activity by anti-m-AAT was observed, nor was anti-s-AAT inhibitory toward m-AAT. Either fraction I or II of m-AAT was, however, completely precipitated by anti-m-AAT prepared with M-III as antigen.

Catalytic Properties. Under the conditions of the assays used here, there would appear to be significant differences in the specific activities of s-AAT and the most highly purified m-AAT fraction III. These values are approximately 240 units/mg of protein for s-AAT and 170 units/mg for M-III. These differences are exaggerated, however, due to the differing sensitivity of the isoenzymes to anions, particularly phosphate and chloride. As seen from Figure 5, increasing concentrations of potassium phosphate have slightly stimulatory effects on s-AAT but are inhibitory toward m-AAT. Taking data at 70 mM phosphate to represent the situation encountered in the normal AAT assay employed here, it can be determined that s-AAT activity is overestimated by approximately 8% whereas m-AAT activity is underestimated by 26%. When these corrections are applied, the specific activities of the two isoenzymes are quite similar.

An additional complicating factor was found when pyridoxal phosphate was added to the assay of either isoenzyme. If phosphate was present at 69 mM concentration, pyridoxal phosphate (10 μM) had no appreciable effect. However, in the absence of phosphate, pyridoxal phosphate was stimulatory, increasing the activity of s-AAT by 30% and m-AAT by 55%. The effect of pyridoxal phosphate does not appear to be one of activating resolved apoenzyme because dialysis against pyridoxal phosphate did not alter this effect. Rather it would seem that pyridoxal phosphate is activating through attachment at sites normally not containing this coenzyme and that this attachment, and the resulting increase in catalytic activity, is blocked by phosphate.

Apparent Michaelis constants for all substrates of AAT are recorded in Table IV. The results shown were obtained in each case with the nonvaried substrate at the concentration stated in the Materials and Methods section, but the Michaelis values determined in this manner were similar to those found when the method of Velick and Vavra (1962) was employed. This latter method uses primary plots of the reciprocals of velocity and variable substrate at different concentrations of fixed substrate, then secondary plots of slopes and intercepts *vs.* the reciprocal of fixed substrate concentration. Both methods yield similar results for the Michaelis constant of a variable substrate when sufficiently high concentrations of fixed substrate are used.

Increasing the concentration of oxaloacetate from 1.4 to 3.9 mM increased the K_m of s-AAT for glutamate from 4.8 to

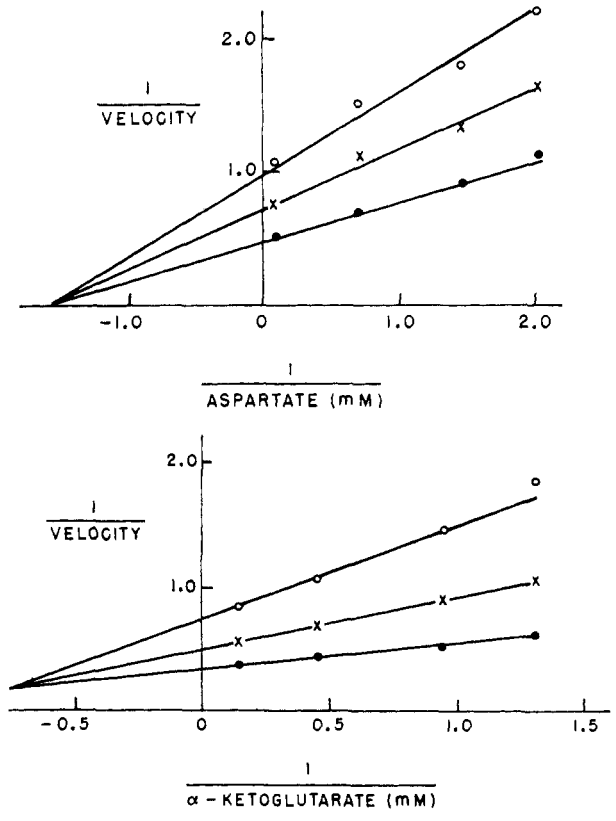

FIGURE 6: Effect of DL-glyceraldehyde 3-phosphate on m-AAT activity. Purified m-AAT, fraction III, was incubated 30 min at 25° with DL-glyceraldehyde 3-phosphate prior to addition of other assay components. Top: inhibition toward aspartate. Bottom: inhibition toward α-ketoglutarate. (●) No glyceraldehyde 3-phosphate; (×) 0.1 mM inhibitor (final concentration); (○) 0.25 mM inhibitor. Velocity is expressed in milliunits.

9.6 mM whereas V_{max} did not change appreciably. A similar effect was noted for the mitochondrial enzyme (fraction III) in that increasing concentrations of oxaloacetate raised the apparent K_m for glutamate; in this case, K_m for glutamate increased from 2.8 to 6.1 mM as oxaloacetate concentrations were varied from 0.56 to 3.8 mM. Thus it appears that oxaloacetate is a mild inhibitor of either enzyme's ability to convert glutamate into α-ketoglutarate. Pyruvate concentrations as high at 17 mM had no detectable effects on the kinetics of the aspartate aminotransferases.

Aside from the predicted effects (Henson and Cleland, 1964) of aspartate and α-ketoglutarate as weak inhibitors (competitive and noncompetitive, respectively, for a Ping-Pong bi-bi mechanism) of glutamate transamination, only one other compound was found to be inhibitory. DL-Glyceraldehyde 3-phosphate was a noncompetitive inhibitor of both α-ketoglutarate, with a K_i of 0.25 mM, and aspartate, with a K_i of 0.15 mM (Figure 6). These results are for m-AAT but inhibitions of a generally similar nature were also observed with s-AAT. These findings with glyceraldehyde 3-phosphate parallel those reported by Kopelovich *et al.* (1970).

Discussion

Molecular Properties of the Purified Enzymes. The procedures described herein for the purification of rat brain s-AAT and m-AAT result in good yields of apparently homogeneous enzymes, except for some slight cross-contamination between m-AAT fractions M-II and M-III. Aged-induced conversions of M-III and M-II into an M-I-type subform and the similar physical properties of all three, however, render unlikely the possibility that such forms differ appreciably in properties other than those concerned with binding of pyridoxal phosphate and catalytic activity. The differing electrophoretic mobilities exhibited by these subforms were not reflected in the amino acid compositions of M-II and M-III, although small differences in the ratio of acidic amino acids to their amide forms detected in the present analyses may be significant. Furthermore, antibody prepared against fraction M-III cross-reacted with both fractions M-II and M-I, ruling out gross differences in amino acid composition.

The cytoplasmic AAT isolated contained no alternate forms, although aging of preparations resulted in the appearance of a second, more rapidly migrating component. Analyses of crude brain homogenates occasionally reveal two forms of s-AAT, and it is presumed that these are identical to the two forms found in aged s-AAT. Failure to observed a second s-AAT on the DEAE-Sephadex chromatographic analysis would suggest that the early purification steps exclude any faster component. In contrast to these findings, beef heart s-AAT purified in a manner analogous to rat brain s-AAT elutes as three components from DEAE-cellulose columns (unpublished observations). This may support the suggestion made below that the subforms are transition states in protein turnover and that the brain enzyme differs in its rate of destruction from the heart enzyme.

The generation of subforms *in vitro* during storage may be representative of an event that occurs *in vivo*. Martinez-Carrion *et al.* (1967) have demonstrated the existence of subforms for both s-AAT and m-AAT in fresh porcine heart muscle and pointed out the similarity in amino acid composition of the cytoplasmic subforms. In addition, their report indicates a similarity in amino acid composition of the mitochondrial subforms, a similarity in substrate affinities and the ability of some of these forms to be converted into others. Thus it is likely that each tissue has the genetic capacity to produce only one of the m-AAT subforms, presumably for brain, the one represented by fraction M-III, and only one s-AAT subform. It is then these parent subforms that give rise to the others. Because the subforms of each type of AAT appear to be interconvertible within the type, certain of these subforms may represent intermediates in the degradation process of AAT. It is possible that the less active subforms are more susceptible to proteolytic attack than the parent AAT and that the subforms generated from the parent AAT are simply transition states in normal protein turnover.

Catalytic Properties and Functions of Brain Aspartate Aminotransferases. An earlier report (Henson and Cleland, 1964) indicated that pig heart cytoplasmic aspartate aminotransferase was not inhibited by oxaloacetate when catalyzing the conversion of glutamate into α-ketoglutarate but both of the rat brain enzymes exhibit some inhibition by oxaloacetate. Apparent Michaelis constants for the substrates of both enzymes are similar to those obtained by other workers under nearly identical conditions of buffer concentration and pH for pig heart (Nisselbaum and Bodansky, 1964) and rat liver (Boyd, 1961; Harpring, 1965). Although there are differences between the Michaelis constants reported by Bonavita (1959) for human brain AAT and those reported here for rat brain, it is difficult to make a strict comparison since the purifications scheme of Bonavita would lead to preferential purification of s-AAT but might not exclude m-AAT. Additionally, there may indeed be a species variations between the s-AAT of the two sources.

The kinetic properties of the brain AAT isoenzymes are sufficiently different with respect to the Michaelis constants for aspartate and α-ketoglutarate that the forms may operate in opposite directions for purposes of the transfer of reducing equivalents from cytoplasm to mitochondria. The presence of malate dehydrogenase and AAT in both mitochondrial and cytoplasmic compartments can in principle result in a shuttle of reducing power as proposed by Borst (1961). Whether such oppositely directed reactions do in fact occur must await detailed information on the concentration of the AAT substrates in both mitochondrial and cytoplasmic fractions. Garfinkel (1970) has simulated certain aspects of glutamate metabolism in brain and has assumed in his best models that the concentration of α-ketoglutarate is almost 10-fold lower in mitochondria than in cytoplasm. Other AAT substrates are nearly evenly distributed. These estimates are therefore consistent with the idea of a role for AAT in the regeneration of NAD^+ during aerobic glycolysis in the cytosol.

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