Optical Rotatory Dispersion of L-Aspartate β-Decarboxylase and Its Derivatives*

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ABSTRACT: The optical rotatory dispersion of aspartate β-decarboxylase and a number of its derivatives has been determined over the range 225–500 mμ. The pyridoxal 5′-phosphate enzyme (holoenzyme) exhibits anomalous rotation in the region of the absorption of the chromophore (maximum, about 360 mμ), while the dispersion of the apoenzyme is plain in this region. The difference dispersion curve (holoenzyme − apoenzyme) exhibits a Cotton effect with a point of inflection at 360 mμ. The magnitude of the trough of rotation at 233–234 mμ is substantially the same for the apoenzyme and holoenzyme. Rotatory dispersion titration of the apoenzyme with pyridoxal 5′-phosphate indicates that about 15.5 moles of pyridoxal 5′-phosphate are bound/800,000 g of enzyme, a value which is in close agreement with spectrophotometric, chemical, and microbiological data. The pyridoxamine 5′-phosphate form of the enzyme exhibits a plain dispersion similar to that of the apoenzyme, while the 4′-deoxypyridoxine 5′-phosphate enzyme exhibits a small Cotton effect with a point of inflection at about 320 mμ. The 4′-deoxypyridoxine 5′-phosphate derivative of porcine glutamate–aspartate transaminase was also prepared and found to exhibit a Cotton effect similar to that of the corresponding aspartate β-decarboxylase derivative. When aspartate β-decarboxylase holoenzyme was reduced with sodium borohydride its maximum absorbancy shifted from 360 to 320 mμ, and the characteristic Cotton effect exhibited by the holoenzyme was replaced by a smaller Cotton effect with a point of inflection at 320 mμ. Derivatives of the holoenzyme of aspartate β-decarboxylase obtained by treatment with sodium cyanide, hydroxylamine, and *threo*-β-hydroxy-DL-aspartate exhibited rotatory dispersion curves that were, within experimental error, the same as that of the apoenzyme. The optical rotatory dispersion curves of aspartate β-decarboxylase and its derivatives are compared with earlier and present data on the corresponding derivatives of glutamate–aspartate transaminase, and the significance of the data is considered in relation to the nature of the cofactor–enzyme linkages.

Aspartate β-decarboxylase has been isolated in apparently homogeneous form from *Alcaligenes faecalis* (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964a) and from *Achromobacter* (Wilson, 1963; Wilson and Kornberg, 1963). Studies with the *Alcaligenes* enzyme led to the conclusion that the enzyme acts both as a relatively nonspecific L-amino acid transaminase and as an L-aspartate β-decarboxylase. The slow transamination reaction with L-aspartate to yield oxaloacetate and the pyridoxamine 5′-phosphate form of the enzyme results in inactivation of the enzyme for aspartate decarboxylation; this inactivation may be prevented by adding a variety of α-keto acids which regenerate the pyridoxal 5′-phosphate form of the enzyme by transamination. In the absence of α-keto acids, pyridoxamine 5′-phosphate dissociates readily, leaving the apoenzyme, which may be reactivated by pyridoxal 5′-phosphate or by pyridoxamine 5′-phosphate plus an α-keto acid. In addition to pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate the apoenzyme can bind the inhibitory cofactor analog 4′-deoxypyridoxine 5′-phosphate (Novogrodsky and Meister, 1964b). Other derivatives of the pyridoxal 5′-phosphate enzyme from *Achromobacter* formed by reaction with sodium borohydride, carbonyl reagents, and the substrate analog β-hydroxyaspartate have also been reported (Wilson and Kornberg, 1963).

In an effort to learn more about the binding of the various vitamin B_6 cofactors and derivatives to the enzyme, we have carried out determinations of optical rotatory dispersion. The binding of a symmetrical chromophoric molecule to a protein may induce anomalous optical rotatory dispersion, a Cotton effect, which is believed to be due to asymmetric orientation on the protein of the otherwise optically inactive chromophore. Spectropolarimetry has only recently been applied to the study of chromophoric proteins; Ulmer and Vallee (1965) have reviewed this field and have summarized its present status.

This paper describes the rotatory dispersion characteristics of aspartate β-decarboxylase and compares these findings with those previously reported for glutamate–aspartate transaminase; the latter enzyme is the only other pyridoxal 5′-phosphate enzyme which has thus far been so studied (Fasella and Hammes,

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1964, 1965; Torchinsky and Koreneva, 1964; Breusov *et al.*, 1964). The large Cotton effect observed with aspartate β-decarboxylase has been used as an indicator in the titration of the apoenzyme with pyridoxal 5′-phosphate. We have also observed smaller Cotton effects with the reduced and 4′-deoxypyridoxine 5′-phosphate derivatives of aspartate β-decarboxylase, and with the 4′-deoxypyridoxine 5′-phosphate derivative of glutamate–aspartate transaminase, a form of the latter enzyme not previously studied in this manner. The significance of the spectropolarimetric data is considered in relation to the nature of the cofactor–enzyme linkage.

Experimental Section

Materials. L-Cysteine sulfinic acid, *threo*-β-hydroxy-DL-aspartic acid, pyridoxal 5′-phosphate, 4′-deoxypyridoxine 5′-phosphate, and pyridoxamine 5′-phosphate were purchased from California Corp. for Biochemical Research. Protamine sulfate, maleic acid, and sodium borohydride were obtained from Nutritional Biochemical Corp., Cleveland, Ohio, Matheson, Coleman and Bell, East Rutherford, N. J., and Metal Hydrides Inc., Beverly, Mass., respectively. DEAE-cellulose was purchased from Brown Co., Berlin, New Hampshire, and prepared for use according to Peterson and Sober (1962). Glutamate–aspartate transaminase (porcine heart), a product of C. F. Boehringer and Soehn (Mannheim, Germany), was purchased from California Corp. for Biochemical Research.

Isolation of Aspartate β-Decarboxylase and Preparation of Its Derivatives

Aspartate β-decarboxylase was isolated from *A. faecalis* by an extensive modification of several previously published procedures (Novogrodsky and Meister, 1964a; Soda *et al.*, 1964; Wilson, 1963). The cells were grown in 580-l. batches on the medium previously described (Novogrodsky and Meister, 1964a) which contains 25 mM sodium succinate as the carbon source and 12.5 mM NH_4Cl as the nitrogen source.[1] The cells were harvested several hours after cessation of the logarithmic growth; such preparations were found to give the highest yields of enzyme. The cells were washed with distilled water and stored as a frozen paste at −70°.

Step I. CELL EXTRACT. Cells (800 g of frozen paste) were suspended in water (final volume, 900 ml) and were then disrupted by treatment for 15 min in a 20 kc MSE ultrasonic disintegrator (Measuring and Scientific Equipment Co., Ltd., London) in 300-ml batches. The extract thus obtained was centrifuged at 20,000*g* for 30 min, the supernatant solution was decanted, and the precipitate was resuspended in a final volume of 300 ml of water and again sonicated and centrifuged. To the combined supernatant solutions were added 0.2 M maleic acid, pyridoxal 5′-phosphate, EDTA, and 2-mercaptoethanol to achieve final concentrations, respectively, of 10 mM, 0.1 mM, 1 mM, and 1 mM; the pH was adjusted to 5.0 by addition of 1 N HCl.

Step II. HEAT TREATMENT. The solution was kept at 50° for 1 hr, after which it was cooled to 15°, and mixed with protamine sulfate (1 g/10 g of protein); the mixture was centrifuged at 20,000*g* for 30 min. The pH of the supernatant solution was adjusted to 7.0 by addition of 4 N KOH.

Step III. DEAE-CELLULOSE CHROMATOGRAPHY. The enzyme was applied to the top of a DEAE-cellulose column (5 × 60 cm) prepared from 180 g of DEAE-cellulose in 0.01 M Tris–maleate buffer, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The column was developed with a linear gradient between 5 l. of this buffer and 5 l. of this buffer containing 0.3 M NaCl. The active enzyme fractions, which were eluted when between 6 and 7 l. of buffer had emerged from the column, were combined (1200 ml), and diluted with 3 volumes of water; this solution was applied to a column of DEAE-cellulose (5 × 6 cm) under 1 psi of pressure, and eluted with Tris–maleate buffer containing 0.5 M NaCl. The active fractions (120 ml) were combined and concentrated to 7.3 ml by ultrafiltration through 0.25-in. diameter dialysis tubing *in vacuo* (Peterson and Sober, 1962) with simultaneous dialysis *vs.* 0.1 M potassium phosphate, pH 6.8.

Step IV. SUCROSE GRADIENT CENTRIFUGATION. The concentrated enzyme was applied to the top of six separate 25-ml linear sucrose gradients (8–25% sucrose in 0.1 M potassium phosphate, pH 6.8). Centrifugation was carried out in the swinging bucket rotor (No. 25) of the Spinco Model L ultracentrifuge at 25,000 rpm for 20 hr. The major protein peak (containing almost all of the activity) was found in the lower half of the tube; this fraction was concentrated by ultrafiltration, followed by dialysis *vs.* 0.1 M potassium phosphate (pH 6.8) to remove sucrose. A representative purification is described in Table I. The enzyme was essentially homogeneous when examined in the analytical ultracentrifuge.[2] Agar gel electrophoresis showed a single minor contaminant. Further studies on the physicochemical characterization of the enzyme will be reported subsequently.

Resolution of the Enzyme. Solutions of the purified enzyme (2–5 mg/ml) were treated with L-aspartate to give a final concentration of 0.1 M and then dialyzed *vs.* 1 l. of 1 M sodium acetate buffer (pH 5.0) containing 0.1 M L-aspartate for 2–3 days with a change of buffer each 24 hr. The enzyme was then dialyzed *vs.* several changes of 0.1 M potassium phosphate (pH 6.8) or 0.1 M sodium acetate (pH 6.8).

Reduction of the Enzyme with Sodium Borohydride. One-tenth volume of a freshly prepared aqueous solu-

[1] Carried out at the New England Enzyme Center, Tufts University School of Medicine.

[2] We are indebted to Dr. Rudy H. Haschemeyer for these determinations. The refractive index increment was determined in the Model E ultracentrifuge using the double-sector synthetic boundary cell and interference optics (Richards and Schachman, 1959).

TABLE I: Purification of Aspartate β-Decarboxylase.[a]

Step	Fraction	Volume (ml)	Protein		Activity	
			Concn (mg/ml)	Total (mg)	Specific[b] (units/mg)	Total (units)
I	Initial cell extract	1,000	27.2	27,200	61.5	1,670,000
II	Heat and protamine-treated extract	830	5.6	4,650	250	1,160,000
III	Concentrated DEAE-cellulose eluate	7.3	25.5	186	2,620	487,000
IV	Sucrose gradient	60	2.26	136	3,240	441,000

[a] Experimental details are given in the text. [b] Micromoles of product formed per hour (units) per milligram of protein under the conditions described in the text.

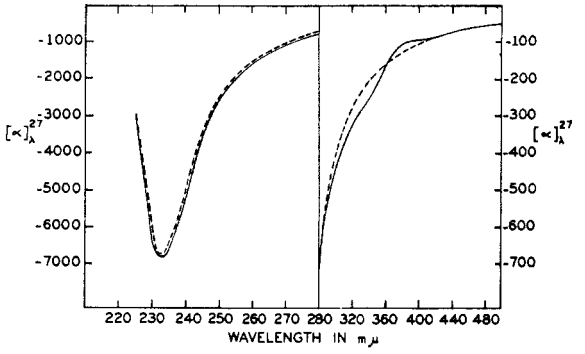

FIGURE 1: Rotatory dispersion curves of aspartate β-decarboxylase (0.1 M potassium phosphate, pH 6.8). Measurements were made in a cell with a 1-cm path length at 9.2 mg/ml in the 300–500-mμ range and at 0.92 mg/ml below 300 mμ: interrupted curve, apoenzyme; solid curve, apoenzyme plus 10^{-4} M pyridoxal 5′-phosphate.

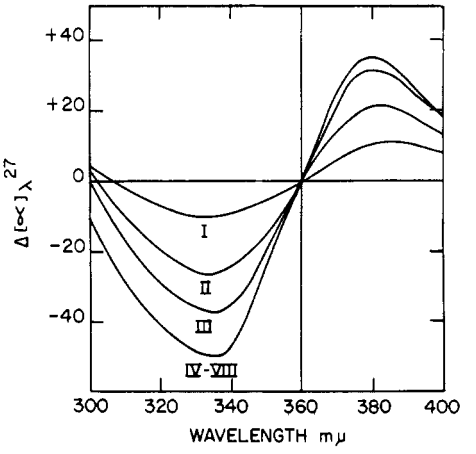

FIGURE 2: Rotatory dispersion titration of aspartate β-decarboxylase apoenzyme with pyridoxal 5′-phosphate. Pyridoxal 5′-phosphate was added to a solution of the apoenzyme [9.9 mg/ml (I–III) or 4.8 mg/ml (IV–VIII)] in 0.1 M sodium acetate (pH 7.0) to give the following ratios of pyridoxal 5′-phosphate to protein (moles of pyridoxal 5′-phosphate added/800,000 g of enzyme): I, 4.15; II, 8.4; III, 12.7; IV, 17.1; V, 21.4; VI, 25.8; VII, 30.1; VIII, 34.4. Rotatory dispersion curves were recorded after 15 min at 23°; path length, 1 cm; difference dispersion curves were calculated after correction for dilution.

tion of sodium borohydride (10 mg/ml) was added to a solution of the enzyme (5–10 mg/ml) in 0.1 M potassium phosphate (pH 6.8) in a centrifuge tube; the tube was immediately centrifuged at 4° for 30 min, at low speed to reduce foaming (Hughes *et al.*, 1962). The solution was then dialyzed overnight *vs.* 0.1 M sodium acetate (pH 6.8).

Resolution of Glutamate–Aspartate Transaminase. The pyridoxal phosphate form of glutamate–aspartate transaminase was resolved (Wada and Snell, 1962) by adjusting a 1% suspension of the holoenzyme in 60% ammonium sulfate to pH 3.0. After standing at 4° overnight the pellet obtained by centrifugation was dissolved in 0.1 M dipotassium hydrogen phosphate and dialyzed *vs.* 0.1 M potassium phosphate (pH 6.8). Assay by the method of Sizer and Jenkins (1962), in which the transaminase reaction is coupled with malate dehydrogenase, showed that the enzyme was 90–96% resolved.

Methods. Enzyme activity was determined as described previously by the colorimetric determination of sulfite liberated from L-cysteine sulfinic acid, which is a substrate for aspartate β-decarboxylase (Soda *et al.*, 1964). The standard assay system consisted of sodium α-ketoglutarate (0.5 μmole), pyridoxal 5′-phosphate (0.5 μmole), sodium acetate buffer, pH 5.0 (350 μmoles), and enzyme in a final volume of 1.0 ml. After preincubation for 10 min at 37°, 0.5 ml of sodium L-cysteine sulfinate (50 μmoles) was added to start the reaction; after 10 min, 0.5 ml of 50% trichloroacetic acid was added. Sulfite was determined by the fuchsin method of Grant (1947); the colors were compared at 550 mμ. During purification, the protein concentration was determined by the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Specific

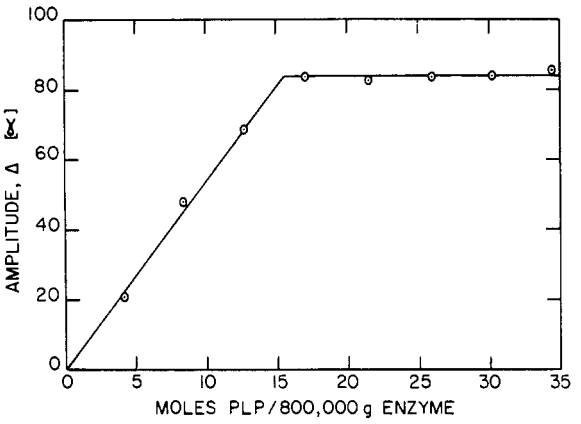

FIGURE 3: Rotatory dispersion titration curve of aspartate β-decarboxylase apoenzyme with pyridoxal 5′-phosphate. The amplitudes of the Cotton effects (determined from the difference dispersion curves given in Figure 2) are plotted *vs.* the moles of pyridoxal 5′-phosphate added/800,000 g of enzyme.

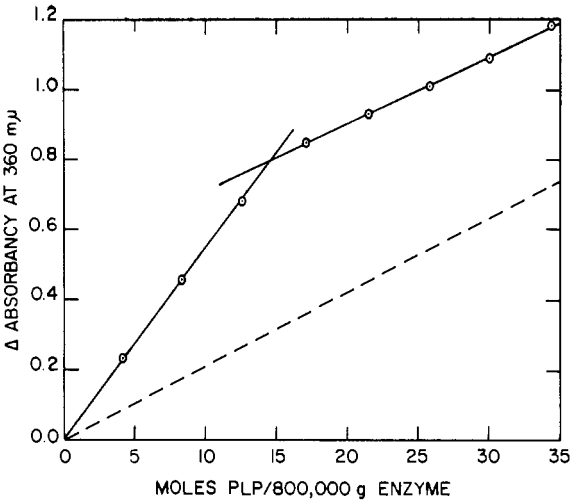

FIGURE 4: Spectrophotometric titration curve of aspartate β-decarboxylase apoenzyme with pyridoxal 5′-phosphate. The spectra were taken following the recording of each rotatory dispersion curve in the experiment described in Figure 2. The difference between the absorbancy at 360 mμ of the apoenzyme plus pyridoxal 5′-phosphate and the absorbancy of the apoenzyme is plotted *vs.* the moles of pyridoxal 5′-phosphate added/800,000 g of enzyme. The dotted line gives the absorbancy at 360 mμ when equivalent amounts of pyridoxal 5′-phosphate were added to a control in which enzyme was omitted.

enzymatic activity is expressed in terms of micromoles of sulfite formed per hour (units) per milligram of protein at 37°.

Spectra of the enzyme were obtained with a Cary Model 14 spectrophotometer. Optical rotation was determined with a Cary Model 60 spectropolarimeter. All determinations were made at 27° on at least two concentrations of sample or in two cells of different path lengths in order to reduce artifacts resulting from light scattering. Protein concentrations were determined spectrophotometrically at 278 mμ; the values were between 0.1 and 10 mg/ml. The extinction coefficient ($E_{278}^{1\%} = 11$) was calculated from determinations made on a solution of purified apoenzyme which had been dialyzed extensively *vs.* 0.1 M potassium phosphate buffer (pH 6.8); the protein concentration was determined refractometrically, by assuming a specific refractive increment of 1.85×10^{-3}.[2]

The observed rotation of each sample was corrected for the rotation of a blank which was identical except that enzyme was omitted. The specific rotation of the sample was calculated from the recorded data at 5- or 10-mμ intervals. The maximum error in the observed rotation is estimated to be about ±0.0005°. Therefore, the error of the specific rotation for most samples (observed rotation, 0.1–0.2°) is less than ±1%. Difference dispersions were obtained by subtracting the specific rotation of the apoenzyme from that of the derivative. The amplitude of the Cotton effect was calculated as the vertical distance in degrees of rotation between the peak and the trough in the difference spectrum.

Results

Studies on Apoenzyme and Holoenzyme. The optical rotatory dispersion curves of aspartate β-decarboxylase in the presence and absence of pyridoxal 5′-phosphate are given in Figure 1. The two curves are superimposable at high and low wavelengths, but the rotation of the holoenzyme is anomalous in the region of the absorption of the chromophore, which has a maximum at 360 mμ. The dispersion of the apoenzyme is plain in this region. The absolute values of the rotation of the enzyme at 233 mμ varied by ±5% with different preparations, but the difference between the rotations of the holoenzyme and apoenzyme at this wavelength when the same preparation of enzyme was used and treated with 0.01 volume of pyridoxal 5′-phosphate was less than ±1%.

When the specific rotation of the apoenzyme is subtracted from that of the holoenzyme, the difference dispersion curve is obtained (Figure 2, curves IV–VIII). This gives the rotation associated only with the bound chromophore; the curve has a shape close to that of an idealized positive Cotton effect where the inflection point is very close to the maximum absorption of the protein-bound pyridoxal 5′-phosphate (360 mμ). The rotatory dispersion of the holoenzyme was measured at protein concentrations of 0.1, 1.0, and 10 mg/ml in cells of path lengths 10, 1.0, and 0.1 cm, respectively, so that rotations of similar magnitude would be recorded. The amplitudes of the observed Cotton effects varied by ±8%; such variation is within the estimated range of experimental error for observed rotations of this magnitude. Thus, the Cotton effect appears to be

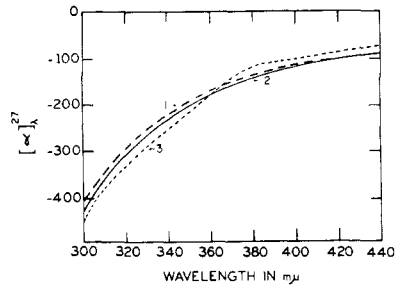

FIGURE 5: Effect of pyridoxamine 5′-phosphate and pyridoxamine 5′-phosphate plus α-ketoglutarate on the rotatory dispersion of aspartate β-decarboxylase. Rotatory dispersion was determined after incubation of a solution of the apoenzyme (12 mg/ml) in 0.1 M potassium phosphate (pH 6.8) containing the indicated additions for 30 min at 37°; path length 0.2 cm: curve 1, apoenzyme; curve 2, apoenzyme plus 1 mM pyridoxamine 5′-phosphate; curve 3, apoenzyme plus 1 mM pyridoxamine phosphate and 1 mM α-ketoglutarate.

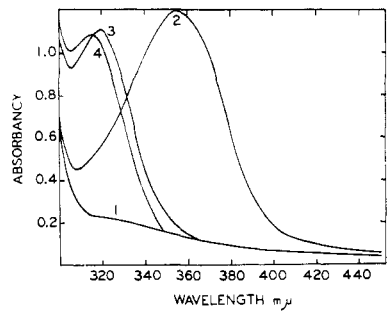

FIGURE 6: Absorption spectra of aspartate β-decarboxylase derivatives. Enzyme concentration is 7.2 mg/ml in 0.1 M sodium acetate (pH 6.8): curve 1, apoenzyme; curve 2, holoenzyme; curve 3, sodium borohydride reduced derivative; curve 4, 4′-deoxypyridoxine 5′-phosphate derivative.

independent of protein concentration over the range studied. The amplitude of the Cotton effect was also independent of pH between 5.7 and 7.7.

The several curves shown in Figure 2 were obtained in an experiment in which the apoenzyme was treated with increasing amounts of pyridoxal 5′-phosphate. In Figure 3, the amplitude of the Cotton effect is plotted *vs.* the moles of pyridoxal 5′-phosphate added/800,000 g of enzyme. [A molecular weight of about 800,000 has previously been estimated for this enzyme (Novogrodsky and Meister, 1964a).] No significant increase in amplitude was observed after the fourth addition of pyridoxal 5′-phosphate. The amplitude is proportional to the amount of pyridoxal 5′-phosphate added until a saturation point is reached at about 15.5 moles of pyridoxal 5′-phosphate/800,000 g of protein. The spectrum of the enzyme was recorded after each of the additions of pyridoxal 5′-phosphate carried out in the experiment described in Figure 2. In Figure 4, the absorbancy at 360 mμ minus the absorbancy of the apoenzyme at this wavelength is plotted *vs.* the moles of pyridoxal 5′-phosphate added/800,000 g of enzyme. This curve changes slope sharply after about 15 moles of pyridoxal 5′-phosphate had been added/800,000 g of enzyme. The slope after the sharp break is close to that of a control in which enzyme was omitted. Although free pyridoxal 5′-phosphate exhibits maximum absorbancy at 388 mμ, it exhibits appreciable absorption at 360 mμ; thus, the curve shown in Figure 4 reflects the presence of both enzyme-bound and free pyridoxal 5′-phosphate. The dotted line represents the absorbancy of free pyridoxal 5′-phosphate.

Studies on Other Forms of the Enzyme. As shown in Figure 5 (curve 2), the rotatory dispersion curve obtained after incubation of the apoenzyme with 1 mM pyridoxamine 5′-phosphate was very similar to that exhibited by the apoenzyme. The available data indicate that pyridoxamine 5′-phosphate is bound to the enzyme under these conditions. Thus, the K_m for pyridoxamine 5′-phosphate is about 0.1 mM, and, in the assay system used here (which contains α-ketoglutarate), pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate are equally effective cofactors when studied at a concentration of 0.3 mM. After incubation of the enzyme with 1 mM pyridoxamine 5′-phosphate followed by dialysis overnight, the spectrum exhibited a small maximum at 325 mμ indicating enzyme-bound pyridoxamine 5′-phosphate; a small peak at 360 mμ was also observed, suggesting that some of the pyridoxamine 5′-phosphate had been converted to pyridoxal 5′-phosphate during dialysis. When the enzyme was incubated with both 1 mM pyridoxamine 5′-phosphate and 1 mM α-ketoglutarate (Figure 5, curve 3) an anomalous dispersion curve characteristic of the holoenzyme was observed.

4′-Deoxypyridoxine 5′-phosphate is an inhibitory analog of pyridoxal 5′-phosphate for both aspartate β-decarboxylase (Novogrodsky and Meister, 1964b) and glutamate–aspartate transaminase (Meister *et al.*, 1954). The spectrum of the 4′-deoxypyridoxine 5′-phosphate derivative of aspartate β-decarboxylase (Figure 6, curve 4) exhibits a maximum at 315 mμ, while that of the corresponding derivative of glutamate–aspartate transaminase is maximal at 320 mμ. The difference dispersion curves for the 4′-deoxypyridoxine 5′-phosphate derivatives of these enzymes are given in Figure 7. Both derivatives exhibited small[3] but definite Cotton effects with points of inflection at about 320 mμ. Under the experimental conditions employed, the amplitude of the Cotton effects varied between 10 and 20° for aspartate β-decarboxylase and between 20 and 30° for glutamate–aspartate transaminase. The variation in amplitudes observed may be ascribed to experimental error inherent in the calculation of small differences between large numbers.

[3] The small Cotton effects observed with the 4′-deoxypyridoxine 5′-phosphate enzyme and the $NaBH_4$-reduced enzyme were not detected under the conditions employed for the studies described in our preliminary report (Wilson and Meister, 1965).

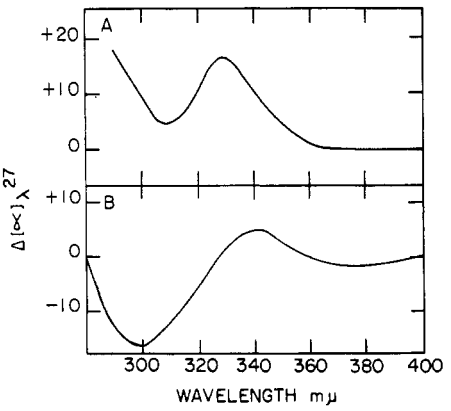

FIGURE 7: Rotatory dispersion difference curves for 4′-deoxypyridoxine 5′-phosphate enzymes. Rotatory dispersion curves of apoaspartate β-decarboxylase (7 mg/ml in 0.1 M sodium acetate, pH 7.0) and apoglutamate–aspartate transaminase (4.4 mg/ml in 0.1 M potassium phosphate, pH 6.8) were recorded in the presence and absence of 2×10^{-4} M 4′-deoxypyridoxine 5′-phosphate; path length, 0.2 cm. The difference dispersions are plotted *vs.* wavelength: A, aspartate β-decarboxylase; B, glutamate–aspartate transaminase.

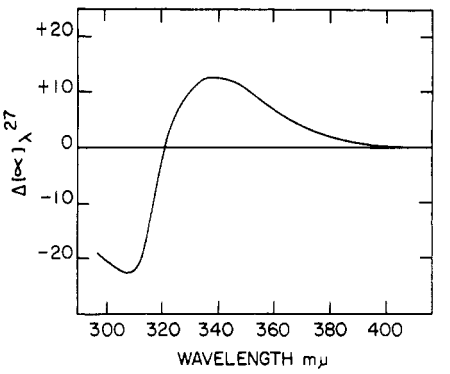

FIGURE 8: Rotatory dispersion difference curve of reduced aspartate β-decarboxylase. Holoenzyme was reduced with sodium borohydride as described in the text. Rotatory dispersion curves of the reduced enzyme and holoenzyme were recorded (5.5 mg/ml in 0.1 M sodium acetate, pH 6.8) and the difference dispersion curve was calculated; path length 1 cm.

When the holoenzyme was reduced with sodium borohydride, its absorption maximum shifted from 355 mμ (Figure 6, curve 2) to 320 mμ (Figure 6, curve 3). The rotatory dispersion difference spectrum of the reduced holoenzyme is given in Figure 8. The data indicate that reduction of the holoenzyme is associated with loss of the characteristic Cotton effect of the holoenzyme, which has an inflection point at about 360 mμ, and with appearance of a smaller Cotton effect with an inflection point at 320 mμ (Figure 8).

Derivatives of the holoenzyme obtained by reaction with sodium cyanide (10 mM), hydroxylamine (1 mM), and *threo*-β-hydroxy-DL-aspartate (1 mM) were also examined. The absorption spectra of these derivatives are similar and exhibit maxima in the range 325–330 mμ; these curves also showed shoulders at 360 mμ suggesting that the several reactions did not proceed to completion. In each case, the rotatory dispersion curves obtained were, within experimental error, identical with that of the apoenzyme. No Cotton effects were observed except for a small Cotton effect with an inflection point at 360 mμ which may probably be ascribed to the presence of unreacted holoenzyme. The findings do not exclude the occurrence of small Cotton effects in the region of 325 mμ, which could not have been detected under the conditions employed. The findings are summarized and compared with data reported in the literature for the glutamate–aspartate transaminase in Table II. For each derivative listed, a rotatory dispersion curve was carried out from 500 to 225 mμ. The magnitudes of the troughs at 233 mμ were identical, within experimental error, with that of the apoenzyme.

Discussion

Aspartate β-decarboxylase may be added to the growing list of chromophoric proteins which exhibit extrinsic Cotton effects. The significance of such Cotton effects has been considered by Ulmer and Vallee (1965), who concluded that extrinsic Cotton effects "are generated in the dispersion curves of proteins upon their asymmetric interaction with a variety of small chromophoric molecules. . ." These effects are superimposable on the background rotation exhibited by the protein and thus do not seem to reflect changes in protein conformation induced by binding of the chromophore. The magnitude of the trough at 233–234 mμ has been often used as a measure of the ordered structure of proteins and is considered to be a sensitive indicator of changes in conformation. In aspartate β-decarboxylase, as in liver alcohol dehydrogenase (Ulmer and Vallee, 1965), the binding of the coenzyme does not change the magnitude of the trough at 233–234 mμ. Fasella and Hammes (1965) reported a difference between the shapes of the ultraviolet rotatory dispersion curves of the holoenzyme and apoenzyme forms of glutamate–aspartate transaminase and the magnitudes of the troughs at 231 mμ. However, the differences are small and the significance of these findings is not yet clear.

Ulmer *et al.* (1961) have previously used rotatory dispersion titration for the quantitative determination of the binding of chromophoric molecules to proteins. Fasella and Hammes (1964) suggested that such rotatory dispersion titration of glutamate–aspartate transaminase might be difficult because of the possibility that pyridoxal 5′-phosphate might bind to the enzyme at sites other than the active sites; however, our data indicate that such titration is possible with aspartate β-decarboxylase. The value obtained here (*ca.* 15.5 moles of pyridoxal 5′-phosphate/800,000 g of enzyme) agrees closely with values obtained by chemical and

TABLE II: Rotatory Dispersion Data for Pyridoxal 5′-Phosphate Enzymes and Their Derivatives.

Enzyme Form	Aspartate β-Decarboxylase			Glutamate–Aspartate Transaminase		
		Cotton Effect				
	$[\alpha]_{233}^{27}$[a] (deg)	Infl pt (mμ)	Amplitude (deg)	Infl pt (mμ)	Amplitude[b] (deg)	Lit. Ref
Apoenzyme	−6870		None		None	
Pyridoxal 5′-phosphate (holoenzyme)	−7190	358	60–85	362 (pH 8.2)	100	Breusov *et al.* (1964)
				430 (pH 4.8)	150	Torchinsky and Koreneva (1963)
Pyridoxamine 5′-phosphate	−6780		None	340	45	Breusov *et al.* (1964)
4′-Deoxypyridoxine 5′-phosphate	−7450	320	10–20	320	20–30	Present work
$NaBH_4$-reduced holoenzyme	−7450	320	35	340	~50	Torchinsky and Koreneva (1963)
NaCN derivative of holoenzyme	−7700		None	340	~65	Torchinsky and Koreneva (1963)
Oxime derivative of holoenzyme	−7500		None		None	Fasella and Hammes (1964)
β-Hydroxyaspartate derivative of holoenzyme	−7700		None	492	45	Torchinsky and Koreneva (1964)
				333	80	

[a] Estimated error ±5%. [b] Estimated from data given in the literature cited.

microbiological procedures (Wilson, 1963; Novogrodsky and Meister, 1964a). The data obtained from rotatory dispersion thus provide a measure of the pyridoxal 5′-phosphate bound to the active sites of the enzyme; although spurious binding to the enzyme may also occur, such interaction is apparently not extensive or associated with detectable changes in optical rotation.

Although detailed chemical explanations of the Cotton effects are not yet possible, the present findings may be considered in relation to suggestions which arose from earlier studies on glutamate–aspartate transaminase. Thus, Snell and Jenkins (1959) postulated that pyridoxal 5′-phosphate is attached to the protein by linkages involving the phosphate group, carbonyl group, and pyridine nitrogen atom. Other studies (Hughes *et al.*, 1962; Wilson and Kornberg, 1963) have indicated that the pyridoxal 5′-phosphate moieties of glutamate–aspartate transaminase and aspartate β-decarboxylase are attached to the respective enzymes by Schiff base linkage between an ε-amino group of the protein and the aldehyde group of pyridoxal 5′-phosphate. Fasella and Hammes (1964) considered the proposal that a three-point attachment of pyridoxal 5′-phosphate to the enzyme is necessary for induction of a Cotton effect, since rupture of the Schiff base linkage by treatment with hydroxylamine abolished the Cotton effect. They also suggested that the Cotton effect observed with the pyridoxamine 5′-phosphate form of glutamate–aspartate transaminase might be due to interaction between the pyridoxamine amino group and the protein. However, it seems pertinent to note the present observation that the 4′-deoxypyridoxine 5′-phosphate derivative of glutamate–aspartate transaminase exhibits a Cotton effect. In addition, Torchinsky and Koreneva (1964) reported that derivatives of glutamate–aspartate transaminase in which the Schiff base linkage to the protein is ruptured by reaction with β-hydroxyaspartate and various carbonyl reagents exhibit Cotton effects.

Although the Cotton effect observed with the pyridoxal 5′-phosphate form of glutamate–aspartate transaminase may be associated with a three-point attachment of the cofactor to the enzyme, it does not follow that the induction of a Cotton effect necessarily requires such binding. It is quite reasonable to suppose that binding of a chromophore at a single enzyme site may induce a Cotton effect. Indeed, the anomalous rotatory dispersion exhibited by both liver and yeast alcohol dehydrogenases in the presence of 1,10-phenanthroline appears to be associated with the formation of an asymmetric enzyme-bound zinc–phenanthrolinate chromophore (Ulmer and Vallee, 1961, 1965). Although the fully coordinate zinc–phenanthrolinate complex is asymmetric and is, therefore, formed as a racemic mixture from free zinc ions, the stereospecific orientation of the zinc atom on the enzyme leads to asymmetric binding of the chromophore to the zinc–enzyme.

The present observations on the 4′-deoxypyridoxine 5′-phosphate derivatives of aspartate β-decarboxylase and glutamate–aspartate transaminase indicate that induction of Cotton effects does not require formation of a covalent link between the vitamin B_6 moiety and the enzyme. Support for this conclusion also comes from the findings cited above by Torchinsky and Koreneva (1964). The absence of Cotton effects with the

cyanohydrin, oxime, and β-hydroxyaspartate derivatives of aspartate β-decarboxylase may indicate that these forms of the enzyme do not possess sufficient asymmetry to induce Cotton effects that can be detected under the experimental conditions employed. It is technically difficult to detect small Cotton effects which exhibit points of inflection appreciably lower than 360 mμ; the sensitivity of the procedure cannot be greatly increased by using higher concentrations of protein because of the high absorbancy of the chromophoric protein. Circular dichroism (Breusov *et al.*, 1964; Johnson and Graves, 1965) may be a more effective tool for further studies in this area.

A striking difference between aspartate β-decarboxylase and glutamate–aspartate transaminase lies in the markedly different optical rotatory dispersions observed with the pyridoxamine 5′-phosphate forms of these enzymes. Thus, no Cotton effect was observed with the pyridoxamine 5′-phosphate form of aspartate β-decarboxylase, while a substantial effect has been reported for the pyridoxamine 5′-phosphate of glutamate–aspartate transaminase. Previous studies have shown that the affinity of glutamate–aspartate transaminase for pyridoxamine 5′-phosphate is about the same as that for pyridoxal 5′-phosphate (K_m 4.4 × 10^{-6} M) (Meister *et al.*, 1954). In contrast, experiments on aspartate β-decarboxylase indicate that the K_m value for pyridoxamine 5′-phosphate is approximately 10^{-4} M or about one thousand times greater than that for pyridoxal 5′-phosphate. Under the conditions of the present studies, a concentration of pyridoxamine 5′-phosphate was used that was sufficient to saturate the apoenzyme. However, the marked differences in the values of K_m for pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate, and the finding that (in contrast to glutamate–aspartate transaminase) the pyridoxamine 5′-phosphate of aspartate β-decarboxylase dissociates readily from the enzyme (Novogrodsky and Meister, 1964a), suggest that there is a significant difference between these enzymes in the manner in which pyridoxamine 5′-phosphate is bound. Presumably, the pyridoxamine 5′-phosphate of the transaminase is bound more tightly and with greater asymmetry than that of the decarboxylase. It seems to be of significance that aspartate β-decarboxylase and glutamate–aspartate transaminase exhibit approximately equal affinity for 4′-deoxypyridoxine 5′-phosphate and that extrinsic Cotton effects of about the same magnitude were observed with both enzymes.

In conclusion, the present work shows that determinations of optical rotatory dispersion can serve usefully in quantitative studies on the binding of pyridoxal 5′-phosphate. Optical rotatory dispersion titration offers a distinct advantage as compared to spectrophotometric titration in that there is no background due to unbound cofactor. Such an advantage would be of greater significance in studies on pyridoxal 5′-phosphate enzymes that exhibit maximum absorbancy at wavelengths relatively close to that of free pyridoxal 5′-phosphate. The present studies also indicate that optical rotatory dispersion is a useful tool for the investigation of the binding of cofactors and cofactor analogs to aspartate β-decarboxylase, and also for the comparison of binding phenomena of various pyridoxal 5′-phosphate enzymes. Optical rotatory dispersion may prove useful in examining the effects of inhibitors that affect coenzyme binding. Thus, dispersion measurements used together with information derived from kinetic, spectrophotometric, and chemical approaches may provide farther insight into the structure and function of the active site of the pyridoxal 5′-phosphate enzymes.

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Protein Synthesis Systems from Rat Brain*

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ABSTRACT: Two distinct cell-free protein synthetic systems have been isolated from brain tissue of 18–21 day old white rats, one based on purified ribosomes and the other on mitochondria. The ribosomal system was typical in its requirements for pH 5 enzymes and an exogenous source of adenosine triphosphate (ATP), and its almost complete inhibition by ribonuclease (RNAase).

The mitochondrial system was one of the most active yet reported. It had no requirement for pH 5 enzymes or exogenous ATP, was not inhibited by RNAase, but was moderately inhibited by 0.5 μmole of dinitrophenol, 1.6 mμmoles of rotenone, or 2 μg of antimycin-A per ml. The activity was sensitive to the concentrations of inorganic phosphate, adenosine diphosphate (ADP), and ATP in the medium. Various neurochemicals had specific effects, notably stimulation by γ-aminobutyric acid in both systems. Proteins from the ribosomal system were fractionated on DEAE-cellulose columns, yielding about 3.5% of the label in a soluble acidic protein fraction. Most of the label, however, was in a protein fraction associated with ribosomal ribonucleic acid (RNA). In presence of an artificial messenger, polyuridylic acid, the system incorporated [^{14}C]-phenylalanine at several times the standard rate, provided the ribosomes were first incubated in a KCl medium low in ATP. Relations of these protein synthetic systems to functional activity of neurons are considered, including a possible role of mitochondria in synthesis of synaptic vesicle proteins.

Two distinct cell-free protein synthetic systems have been isolated from immature rat brain, one localized in purified ribosomes and one in mitochondria. These systems, both highly active, differ in various biochemical properties. The purpose of this paper is to describe and contrast the properties of these two systems, as a contribution to an eventual understanding of functional aspects of protein synthesis in brain.

Ribosomal System

There have been several recent studies of ribosomal protein synthetic systems from brain, including those of Zomzely *et al.* (1964) on rat, of Rubin and Stenzel (1965) on rabbit, and of Murthy and Rappoport (1965a) on rat. A cell-free microsomal system from guinea pig brain has been characterized by Satake *et al.* (1964).

Experimental Procedures

We used immature rats in view of reports (Murthy and Rappoport, 1965b) that immature rat brains provided more active enzyme extracts. The rats were male, Sprague–Dawley strain (Simonsen Laboratories, White Bear Lake, Minn.), 18–21 days old, approximately 45 g. The preparation of ribosomes followed that devised by Munro *et al.* (1964) for the liver system, with a fractionation procedure as outlined in Figure 1. Electron micrographs of the ribosomal preparations, for example, Figure 2, show a fairly high proportion of double ribosomes as well as some larger aggregates. Ultracentrifuge data on this point will be discussed later.

The standard ribosomal system is summarized in Table I. Ribosomes (0.6–0.7 mg of protein) and pH 5 enzymes (1.0–1.5 mg of protein) were separately suspended in medium M. The ATP[1] and GTP, pH 5 enzymes, and ribosomes were added in that order to medium M in 13 mm × 10 cm tubes held in a constant temperature block, and the run was started by addition of labeled [^{14}C]leucine. The reaction was stopped after

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[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; GABA, γ-aminobutyric acid; ACH, acetylcholine; EPI, epinephrine; NEPI, norepinephrine; DOPA, *o*-dihydroxyphenylalanine; CPZ, chloropromazine; SERO, serotonin; α-KGA, α-ketoglutarate; DPN, 2,4-dinitrophenol; GDP, guanosine diphosphate; IDP, inosine diphosphate.