ACKNOWLEDGMENTS

We are grateful to Messrs. Louis Costello and Fred Castillo, and to Mrs. Shirley White for their assistance in various aspects of this work.

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An Optical Rotatory Dispersion Study of Aspartic Amino Transferase*

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Received October 23, 1963

An optical rotatory dispersion study of several forms of the enzyme aspartic amino transferase has been carried out in the wavelength range of 300–600 mμ. The pyridoxal and pyridoxamine enzymes in aqueous solution display anomalous rotatory dispersion indicating a strong interaction between coenzyme and enzyme. When ketoglutarate is added to the aldimine enzyme to form a complex, the rotatory dispersion curve is unchanged. On the other hand, addition of hydroxylamine, yielding an oxime, causes complete disappearance of the Cotton effects. The apoenzyme and the denatured enzyme also have plain dispersion curves. The b_0 for the oxime enzyme is quite high (−490°) suggesting a highly ordered structure, whereas the apo- and native enzymes have quite low b_0 values (> −100°). However, the a_0 values for the oxime and apoenzyme are essentially identical (∼ −220°), while that for the enzyme in urea is markedly changed (−490°). According to current theories, this seems to indicate that pyridoxal phosphate has a profound ordering effect on the enzyme structure.

The use of optical rotatory dispersion for providing information about the conformation of proteins and about their interaction with prosthetic groups is now well established (Urnes and Doty, 1961). The enzyme aspartic amino transferase can exist in several distinct forms (Jenkins and Sizer, 1960; Lis *et al.*, 1960). At high *p*H values (>8) an active enzyme containing tightly bound pyridoxal phosphate and having an absorption maximum at 362 mμ is formed. At lower *p*H values (<5) the enzyme is inactive and its spectral maximum shifts to 430 mμ. In both cases the aldehyde group of the pyridoxal phosphate forms an internal Schiff base with an ε-amino group of the protein (Turano *et al.*, 1961; Hughes *et al.*, 1962). These two forms are jointly termed the *aldimine enzyme*. The active aldimine enzyme can react with amino acid substrates to give keto acids and a protein-containing pyridoxamine phosphate with an absorption maximum at 333 mμ (the aminic enzyme) (Jenkins and Sizer, 1960; Lis *et al.*, 1960). The inactive apoenzyme can be prepared by complete removal of the pyridoxal phosphate. (Banks and Vernon, 1961; Wada and Snell, 1962). The aldimine enzyme can also react with dicarboxylic acids and carbonyl reagents, which are competitive inhibitors of the enzyme reaction, to form inactive complexes (Polyanovsky and Torchinsky, 1961; Velick and Vavra, 1962b; Jenkins and Sizer, 1963; Banks *et al.*, 1963; Karpeisky *et al.*, 1963; Hammes and Fasella, 1964).

An investigation of the optical rotatory dispersion of the various forms of the enzyme is of interest to obtain further information about the apoprotein-coenzyme and enzyme–quasi substrate interactions and to detect possible conformation changes occurring in the protein moiety of the enzyme when it reacts with coenzyme, substrate, and substrate analogs.

EXPERIMENTAL

Aspartic amino transferase from pig hearts was prepared according to Lis (1958). This preparation is homogeneous in the ultracentrifuge and in zonal and free-phase electrophoresis (Passalacqua, 1961). The pyridoxamine form of the enzyme was prepared according to Lis *et al.* (1960). The apoenzyme was prepared by the procedure described by Wada and Snell (1962) for the resolution of transaminases. The apoenzyme thereby obtained had less than 5% of the original activity. The activity could be restored (more than 90% of the original values) after 30 minutes' incubation with 10^{-4} M pyridoxal phosphate at *p*H 8.05 in 0.05 M potassium phosphate buffer at 20°. The denatured enzyme was prepared by putting the pyridoxal enzyme into a buffered solution saturated with recrystallized urea at 20° (9 M). This solution

* This work was supported by a grant (MG-07803) from the National Institutes of Health, U. S. Public Health Service.

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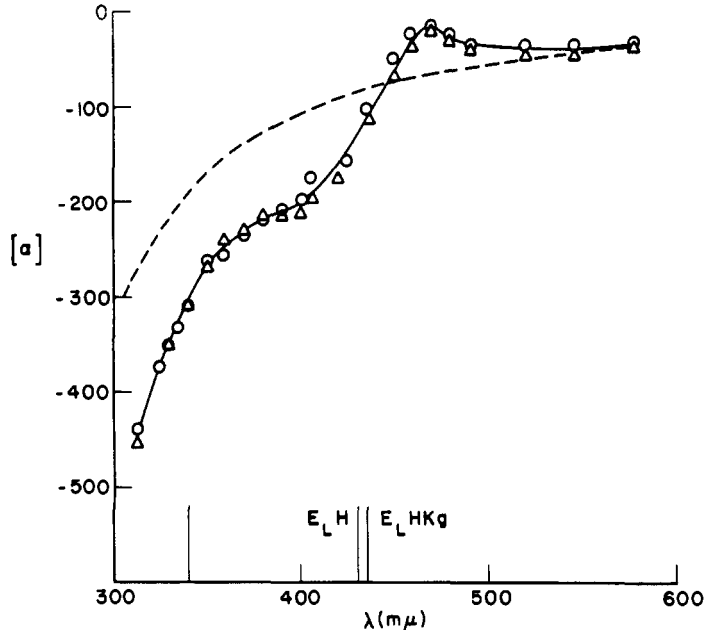

FIG. 1.—Optical rotatory dispersion curves of aldimine enzyme, *p*H 4.95, 0.2 M acetate buffer. O, enzyme alone; Δ, enzyme plus 10^{-2} M ketoglutarate. The long vertical lines on the wavelength scale designate the absorption maxima. The dashed curve is the rotatory dispersion curve of the apoenzyme.

was diluted to a final urea concentration of 8 M and incubated at room temperature for 60 minutes. All other reagents were standard reagent grade. Distilled deionized water was used to prepare all solutions.

Optical rotation was measured by means of a Rudolph Model 200 S-80Q photoelectric spectropolarimeter with an oscillating polarizing prism. The light source was a high-intensity high-pressure mercury lamp (AH 6, General Electric Co.). Excellent stability was obtained by circulating cool deionized water through the quartz-jacketed lamp housing. Standard 5-cm semimicropolarimeter cells with quartz end plates were used in all determinations. The cell housing was thermostated at 12° ± 1.5°. A stream of nitrogen was directed onto the polarimeter-cell end plates to prevent fogging. The oscillating symmetrical angle was 5° and the slit width was less than 0.3 mm. Within the wavelength region examined the absorbancy of the sample in the 5-cm pathway was always less than 1.5. Protein concentrations were varied between 0.1 and 0.5 g/100 ml and were determined spectrophotometrically by measuring the absorption at 280 mμ. Several different concentrations were used to determine the curve for each sample so as to insure the absence of light-scattering effects. Blanks were run on solutions identical to the experimental sample except that they did not contain the enzyme.

The optical rotatory power, $[\alpha]_\lambda$ was calculated from the usual equation

$$[\alpha]_\lambda = \frac{100}{lc}\,\alpha_\lambda$$

where l is the cell length in decimeters, c is the concentration of optically active solution in g/100 ml, and α_λ is the observed rotation corrected for the solvent blank. The maximum error in α_λ, as judged by the reproducibility and estimates of experimental error, is about ±3%. The following solutions were investigated: aldimine enzyme (E_L) at *p*H 8.05, 0.2 M potassium phosphate buffer; aldimine enzyme (E_LH) at *p*H 4.95, 0.2 M sodium acetate buffer; pyridoxamine enzyme (E_M) at *p*H 4.95, 0.2 M acetate buffer; E_LH and ketoglutarate (10^{-2} M) at *p*H 4.95, 0.2 M acetate buffer; E_L and hydroxylamine (10^{-3} M) at *p*H 8.05, 0.2 M phosphate buffer; apoenzyme at *p*H 8.05, 0.2 M phosphate buffer; and denatured enzyme in 8 M urea at *p*H 8.05, 0.2 M phosphate buffer. At the concentrations of ketoglutarate and hydroxylamine used, the enzyme is essentially saturated (Hammes and Fasella, 1964).

Results and Treatment of Data

The optical rotatory dispersion curves of the aldimine enzyme at moderately alkaline and acidic *p*H values in the presence and absence of ketoglutarate and hydroxylamine and of the aminic form of the enzyme are reproduced in Figures 1–4. The rotatory dispersion curve of the apoenzyme is displayed in each figure for reference. The aldimine enzyme in the presence of hydroxylamine, the apoenzyme, and the denatured enzyme gave plain dispersion curves within experimental error in the wavelength region investigated. The data in these cases were treated according to the usual equation (Moffit and Yang, 1956):

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 + \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (1)$$

where

$$[m']_\lambda = [\alpha]_\lambda \frac{\bar{M}}{100}\,\frac{3}{n^2 + 2}$$

$\bar{M}$ is the mean amino acid residue weight, which was taken as 115, n is the refractive index, λ is the wavelength in Angstroms; λ_0 was taken as 2120 A, and a_0 and b_0 are constants to be determined from the data. The constants a_0 and b_0 were obtained by plotting $[m']_\lambda(\lambda^2 - \lambda_0^2)$ versus $1/(\lambda^2 - \lambda_0^2)$. The plots of the data are shown in Figure 5. The refractive index was taken as 1.334 for all solutions except 8 M urea, for which a refractive index of 1.400 was used (Schellman, 1958). The values of a_0 and b_0 found are reported in Table I, together with estimates of the experimental uncertainty. The Cotton effects with other forms of the enzyme extend over such a wide range of wavelength that a precise evaluation of rota-

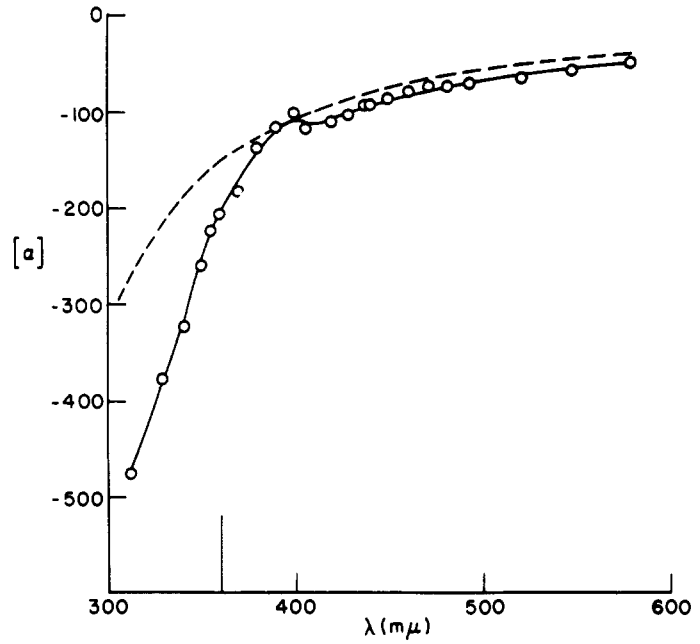

FIG. 2.—Optical rotatory dispersion curve of aldimine enzyme, *p*H 8.05, 0.2 M phosphate buffer. The long vertical line on the wavelength scale designates the absorption maximum. The dashed curve is the rotatory dispersion curve of the apoenzyme.

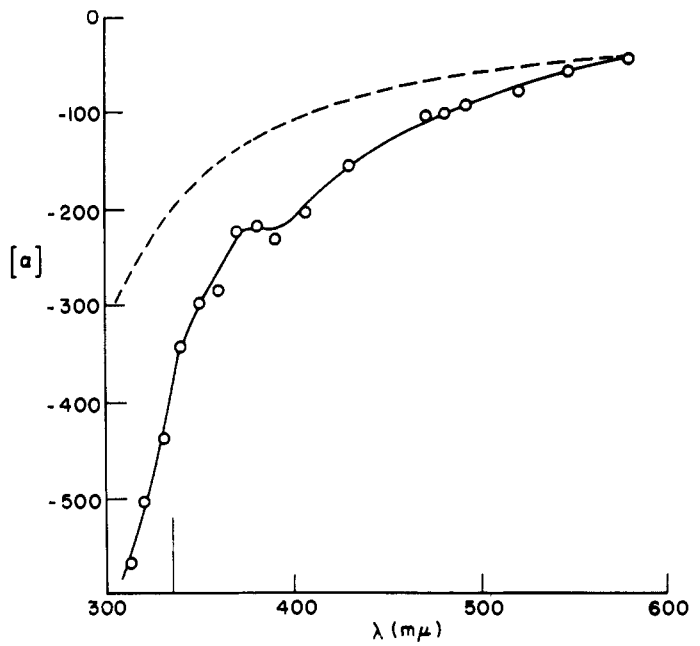

FIG. 3.—Optical rotatory dispersion curve of pyridoxamine enzyme, *p*H 4.95, 0.2 M acetate buffer. The long vertical line on the wavelength scale designates the absorption maximum. The dashed curve is the rotatory dispersion curve of the apoenzyme.

tory dispersion constants a_0 and b_0 is not possible. In passing, it was noted that the results obtained were somewhat dependent on temperature and the particular buffer used.

DISCUSSION

Examination of Figures 1–3 indicates that the optical rotatory dispersion of these forms of the enzyme is anomalous. We shall first consider these anomalies and then examine the behavior of the plain curves.

At *p*H 5 the aldimine enzyme displays two distinct positive Cotton effects. The Cotton effect having a peak at 470 mμ and a negative shoulder at 390–400 mμ probably corresponds to the strong absorption band at 430 mμ. The second effect is less evident,

TABLE I
SUMMARY OF ROTATORY DISPERSION CONSTANTS[a]

Enzyme Form	a_0	b_0	Helix (%)
Apoenzyme	−215 ± 10	−90 ± 10	4
Denatured enzyme	−490 ± 20	−65 ± 10	0
E_L − NH_2OH	−225 ± 10	−490 ± 20	68

[a] See text for experimental conditions.

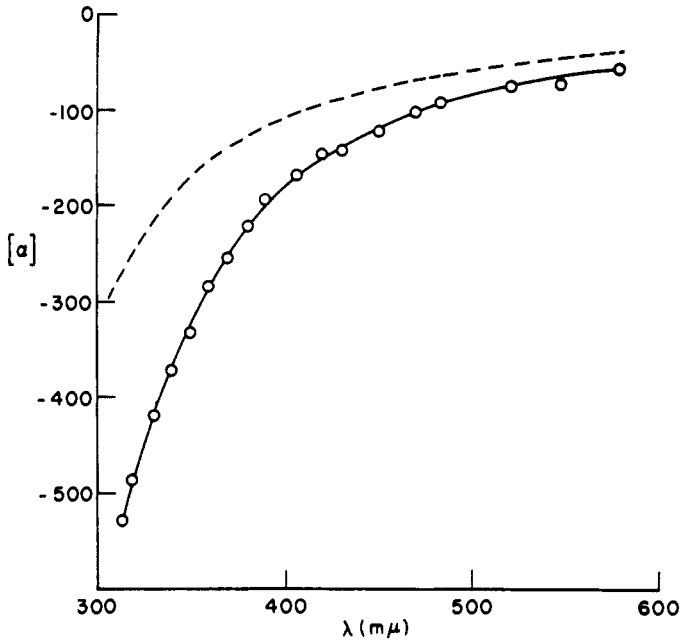

FIG. 4.—Optical rotatory dispersion curve of oxime enzyme (aldimine enzyme + 10^{-3} M NH_2OH), *p*H 8.05, 0.2 M phosphate buffer. The dashed curve is the rotatory dispersion curve of the apoenzyme

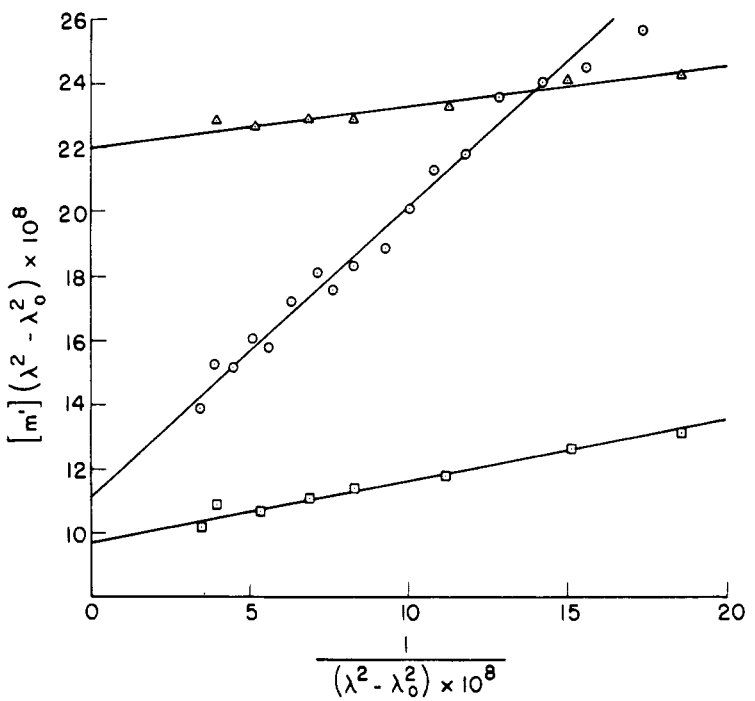

FIG. 5.—Plot of rotatory dispersion data according to equation (1). O, oxime enzyme (aldimine enzyme + 10^{-3} M NH_2OH), *p*H 8.05, 0.02 M phosphate buffer; □, apoenzyme, *p*H 8.05, 0.2 M phosphate buffer; and Δ, denatured enzyme, 8 M urea, *p*H 8.05, 0.2 M phosphate buffer.

partially because it is superimposed on a portion of the background curve where the rotatory power is absolutely much greater. Nevertheless, a slight positive shoulder seems visible in the 360–380 mμ region, while the steepness of the curve in the 315–330 mμ region suggests that a negative trough or shoulder would appear at around 300 mμ. These anomalies are centered about the absorption peak of the enzyme at 335 mμ.

If similar considerations are applied to the curve for the aldimine enzyme at *p*H 8, a small Cotton effect with a positive peak at 400 mμ and a negative shoulder in the 320-mμ region appears to be present corresponding to the 362-mμ absorption peak displayed by the enzyme at this *p*H.

The aminic form of the enzyme also displays a slight Cotton effect with a positive peak around 365 mμ and a negative shoulder, presumably around 300 mμ. This Cotton effect corresponds to the absorption peak shown by the pyridoxamine enzyme at 333 mμ. That the observed Cotton effects are not artifacts (Urnes and Doty, 1961) was proved by the fact that the same values of $[\alpha]_\lambda$ were obtained from experimental measurements at different concentrations of the

enzyme. Although some of the Cotton effects are small, the deviations of the data in Figures 1–3 from plain dispersion curves are definitely outside experimental error. Moreover, none of the curves fit a simple one-term Drude equation or equation (1). In point of fact, such plots tend to accentuate the Cotton effects and make them more noticeable. Unfortunately a stoichiometric optical rotation titration of the apoenzyme with coenzyme is not possible because reconstitution of the enzyme may be accompanied by the binding of coenzyme at binding sites other than those occupied in the native enzyme (Meister, 1955).

The absence of Cotton effects in the apoenzyme dispersion curve and the correspondence between the characteristic wavelengths of the Cotton effects and the wavelength of the coenzyme-absorption peaks indicates that the anomalies are produced by the bound vitamin B_6 derivatives. Since the coenzymes themselves are not optically active, the optical asymmetry must be produced by binding to the apotransaminase. This situation is analogous to that found with liver alcohol dehydrogenase where Cotton effects appear upon binding of coenzyme or inhibitors to the apoprotein (Ulmer *et al.*, 1961).

Denaturation of the enzyme with urea causes the disappearance of the Cotton effect given by the coenzyme chromophore even though spectral evidence indicates that the coenzyme is still partially bound to the protein. This again is not surprising since an ordered structure of the protein is necessary to create the specific disposition in space of the apoenzyme groups capable of binding the coenzyme asymmetrically. (Ulmer *et al.*, 1961). A similar situation was found for dye-helical polypeptide interactions (Stryer and Blout, 1961).

A three-point attachment of pyridoxal phosphate to apoenzyme has been proposed by several workers on the basis of various pieces of evidence (Snell and Jenkins, 1959; Fasella *et al.*, 1961). The three postulated points of interaction on the coenzyme molecule are the phosphate group, the carbonyl group, and the ring nitrogen. In this connection it should be noted that the Cotton effects related to the coenzyme vanish when the aldimine enzyme reacts with hydroxylamine to form a complex absorbing at 370 mμ (See Figs. 4 and 5. Some scatter in the experimental data shown in Fig. 5 exists, but no Cotton effects are apparent.) The observed plain curve is probably due to the fact that an oxime is formed between the aldehyde group of the coenzyme and hydroxylamine, thus destroying one of the coenzyme-enzyme interaction points.

The aldimine enzyme–ketoglutarate complex has a dispersion curve essentially identical to that of the free aldimine enzyme (Fig. 1). This suggests that no major conformation change in the protein or coenzyme has occurred. This is consistent with the fact that the rate of formation of this complex is essentially diffusion controlled (Hammes and Fasella, 1964). It has been suggested that ketoglutarate binds to the coenzyme via the phenolic group or the ring nitrogen (Velick and Vavra, 1962a). However, the optical rotatory dispersion data suggest that the binding must occur without appreciably affecting the protein-coenzyme relationship since the Cotton effects in the absence and presence of ketoglutarate are identical within experimental error. The observation that the inactive forms of the enzyme display the largest Cotton effects is probably related to the fact that further stabilization of the internal Schiff base may occur due to the protonation occurring at the active site (Martell, 1963; Johnston *et al.*, 1963).

Since the rotatory dispersion curves of the denatured enzyme, apoenzyme, and oxime enzyme are plain within experimental error, they are completely described by equation (1) with the constants given in Table I. Included in Table 1 are estimates of the apparent helix content of the protein assuming the denatured protein has no ordered (helical) structure and a completely helical molecule has a b_0 of -630 (Urnes and Doty, 1961). Actually these numbers have essentially no theoretical significance, but are included as a matter of possible interest.

The oxime enzyme has a highly negative b_0 (-490) suggesting, according to current theories (Urnes and Doty, 1961), that this form of the enzyme has a large helix content ($\sim$70%) (or alternatively, simply that the structure is highly ordered). In 8 M urea the value of b_0 drops to $-60°$, indicating that essentially complete breakdown of helical or ordered structures has occurred. Whether or not such an analysis is valid for proteins is questionable and therefore these conclusions must be viewed with considerable caution.

The apoenzyme has a relatively small b_0 although the value of a_0 is similar to that found for the aldimine-hydroxylamine complex. Its optical rotatory dispersion properties, therefore, seem to fall into the "β lactoglobulin type" of globular proteins (Tanford *et al.*, 1960). Several explanations have been proposed for the optical rotatory characteristics of this group of proteins (Tanford *et al.*, 1960; Urnes and Doty, 1961), but in this case simply a lack of helical or ordered structure is most likely responsible for the low b_0. Since a_0 (which is a rough measure of solvent–amino acid interactions) is unchanged from the holoenzyme, it seems unlikely that contributions from α helices with different screw senses or a combination of α and β structures are causing the low b_0 values. In any case, the binding of the coenzyme apparently causes an amazing change in the protein conformation. However, the possibility that an undetected low-wavelength Cotton effect is distorting the value of b_0 obtained for the oxime enzyme should be kept in mind. Again, all of the aforementioned cautions apply to this analysis.

Unfortunately a detailed analysis of the other rotatory dispersion curves in terms of the a_0-b_0 constants cannot be made. A study of the ultraviolet rotatory dispersion curves may shed further light on this problem and will be undertaken in the near future. Preliminary studies of the 233 mμ trough are in accord with the large b_0 values suggested for the pyridoxal enzyme.

We are greatly indebted to Dr. Bert Vallee for allowing us the use of his spectral polarimeter and general laboratory facilities and to Dr. Gerald Fasman for allowing us to make some preliminary measurements of ultraviolet rotatory dispersion curves with his recording instrument.

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Aldolase Dissociation into Subunits by Reaction with Succinic Anhydride

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Received November 26, 1963

When rabbit muscle aldolase (M_w = 142,000) is reacted with succinic anhydride under neutral conditions, the enzyme readily dissociates into three subunits having a molecular weight (M°_w) of 54,500 and a sedimentation coefficient ($s^\circ_{20,w}$) of 2.48 S. This dissociation is accompanied by a shift in the wavelength of maximum absorption from 279.9 to 276.5 mμ and a concomitant decrease in the extinction coefficient ($E^{0.1\%}_{1\ cm}$). Succinylation also produces a large increase in intrinsic viscosity, [η], from 4.04 to 12.80 ml/g and a significant increase in the frictional ratio, f/f_0, from 1.13 to 2.29. Pycnometric analyses at 20° reveal that succinyl aldolase in Tris-KCl buffer (*p*H 8.0, μ = 0.55) has an apparent specific volume of 0.704 ± 0.008 ml/g. Native aldolase in the same medium has an apparent specific volume of 0.745 ± 0.006 ml/g. When succinyl aldolase subunits are exposed to borate-KCl buffer (*p*H 12.5, μ = 0.52), further dissociation occurs and six polypeptides, having apparently equivalent molecular weights (M°_w = 27,000), are obtained per mole of enzyme. N-Terminal amino acid analyses by Edman degradation yield a minimum of four prolines per mole of native aldolase. Similar amino acid analyses on alkali-treated succinyl subunits show no N-terminal groups, indicating that the six subunits do not result from peptide bond cleavage.

Several reports concerning the structure of muscle aldolase indicate that the native molecule is composed of at least three subunits (Kowalsky and Boyer, 1960; Stellwagen and Schachman, 1962; Deal *et al.*, 1963). Recent investigations by Hass and Lewis (1963), however, show that after exposure to alkaline conditions above *p*H 12.0 the enzyme dissociates into six polypeptide chains having apparently equivalent molecular weights. This finding led to the conjecture that alkali might cause the cleavage of specific covalent bonds while promoting disaggregation of highly negatively charged molecules. Consequently, interest was developed in other modifications which might cause subunit formation through the production of negatively charged peptides under neutral conditions.

Maurer and Lebovitz (1956) and Habeeb *et al.* (1958) have shown that succinic anhydride readily reacts with proteins under relatively mild conditions. As a result, a high negative-charge density is imposed upon the molecule through the elimination of $-NH_3^+$ groups and the introduction of $-COO^-$ ions. When this occurs there is a considerable expansion of molecular structure and, as recently shown with hemerythrin (Klotz and Keresztes-Nagy, 1963), dissociation into subunits is possible.

This report is concerned with the dissociation of rabbit muscle aldolase by reaction with succinic anhydride. The resulting succinyl subunits have been examined at different *p*H values and ionic strengths, and several of their physical properties have been described. N-Terminal amino acid analyses have been performed on native and alkali-treated succinyl aldolase in an effort to establish the actual number of monomeric subunits comprising the native molecule.

Materials and Methods

Materials.—Twice-crystallized aldolase was prepared from rabbit muscle by the method of Taylor *et al.* (1948) as modified by Kowalsky and Boyer (1960). Large preparations of the enzyme were stored at 4° in 0.5 saturated ammonium sulfate. The concentration of dissolved aldolase was determined by absorption at 280 mμ using an extinction coefficient, $E^{0.1\%}_{1\ cm}$ = 0.91 (Baranowski and Niederland, 1949).

Succinic anhydride (mp 120°) was obtained from Eastman Kodak Distillation Products Industries, Rochester, N. Y., and was used without further purification.

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