Structural Studies of Paramyosin. II. Conformational Changes*

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The conformation of paramyosin in 0.3 M KCl and in the denaturing solvent GU (5 M guanidine hydrochloride, 1.2 M urea, 0.3 M KCl) was studied by optical rotatory dispersion. b_0 from the Moffitt plot increased from $-570°$ in pH 7.4, 0.3 M KCl to $-165°$ in GU at room temperature; thus, about one-third of the α-helical structure is retained in this solvent. Upon heating of paramyosin in GU, the second stage unfolding of the molecule to a random conformation occurs. If the GU solution is not heated above 60° C, this unfolding is reversible. The unfolding is slightly pH-dependent, as there is a small (6°) difference in transition temperature between acid and neutral GU solutions, which indicates that carboxyl-carboxyl interactions may help to maintain the structure of the partially unfolded molecule. Most of the stabilization of the native protein probably arises from interactions between the nonpolar groups.

A consideration of the optical rotatory properties of paramyosin has led several workers (Cohen and Szent-Györgyi, 1957; Kay and Bailey, 1959; Harrap and Doty, 1960) to postulate that the native molecule exists mainly in an α-helical conformation. However, the conformation in the denatured state is not so well defined. Both Cohen and Szent-Györgyi (1957) and Harrap and Doty (1960) find that some α-helical structure seems to be retained in 9.5 M urea, whereas Kay and Bailey (1959) suggest that complete denaturation occurs in 8 M urea.

Since GU (5 M guanidine hydrochloride, 1.2 M urea, 0.3 M KCl) frees the tyrosyl groups of their interactions, yet apparently does not change the rodlike character of the molecule (Riddiford and Scheraga, 1962), the molecular conformation of paramyosin in this solvent was investigated. There appeared to be only partial denaturation at 25° C; consequently, the optical rotatory properties were studied as a function of temperature and pH.

EXPERIMENTAL

Materials.—The protein preparation used is described in the accompanying paper (Riddiford and Scheraga, 1962). The pH 7.4 and pH 10.5 solutions of paramyosin in 0.3 M KCl were prepared by dissolving the lyophilized protein in water, then dialyzing against 0.3 M KCl, 0.01 M pH 7.4 phosphate buffer for 24 hours. A known volume of base was added to a solution of known concentration to give the desired pH. Because of solubility problems in the acid region, the pH 2.0 solution was prepared by dialyzing a protein solution against 0.3 M KCl, 0.01 N HCl for 36 hours. The concentration was determined from the absorption at 277 mμ, assuming the extinction coefficient to be the same at pH 2 as at pH 7.

Since the extinction coefficient is not known in GU and since there is a large volume increase upon solution of the components of GU, the following method was devised to prepare GU solutions of known concentration. Five ml of paramyosin in 0.3 M KCl was added to a weighed amount of guanidine and urea in a 10-ml volumetric flask. After the GU was dissolved, a known amount of acid or base in 0.3 M KCl was added to give the desired pH. Then 1.5 M KCl was added to make 10 ml of GU solution approximately 0.3 M KCl containing a known concentration of paramyosin. The GU solutions were made at room temperature except for one set prepared at 4° C. Immediately after the pH reading was taken, they were placed in a cold room at 4° C and used either after 30 minutes or after 8–12 hours.

Apparatus.—The Rudolph photoelectric polarimeter Model 200 equipped with a quartz monochromator and an oscillating polarizer was used for optical rotatory dispersion measurements. The light source was a Xenon compact arc lamp which enabled an investigation of the wave length range from 290 to 700 mμ. The symmetrical angle was always 5°.

The solutions to be measured were contained in a water-jacketed 5-cm quartz polarimeter tube of 7 mm bore with fused quartz end-plates manufactured by Optical Cell Co. (Kensington, Md.). Water was circulated through the jacket from a water bath kept at a constant temperature $\pm 0.1°$ C except at temperatures above 50° C, when the variation in temperature was as much as $\pm 1°$ C.

The indices of refraction, n, of the solvents used were determined with the Abbe refractometer at 589 mμ and at 27° C. No correction of n for wavelength or temperature was made.

The TTT1 Radiometer pH meter was used for all pH measurements after being calibrated with standard phthalate, phosphate, and borate buffers prepared as described by Bates (1954). The absorp-

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tion at 277 mμ was measured with a Beckman DU spectrophotometer.

Method.—The cold solution was placed in the polarimeter tube maintained at 5° C and allowed to come to thermal equilibrium. The optical rotation was then measured at the following wavelengths (in mμ): 700, 650, 600, 589, 546, 500, 460, 420, 400, 380, 360, 350, 330, 310, and 300; and, if possible, 295 and 290. After a set of measurements was made, the temperature was raised at the approximate rate of 1° per minute, and every 5° the solution was allowed to equilibrate for 5 to 10 minutes, after which α_{589} was measured. At the temperatures at which the dispersion measurements were made, the solution was allowed to equilibrate for 30 minutes, and α_{589} readings were taken before and after all the dispersion measurements. To test reversibility of the observed changes, after a total of approximately one hour at the high temperatures the solution was cooled at a maximum rate of 3° per minute by cooling the bath with 0° C water. At least 5 hours, and usually a whole night, were allowed before another dispersion curve was made at 5° C. Solvent blanks were obtained in the same manner.

Calculations.—Since the blank rotations varied randomly with temperature, the average rotation obtained at each wavelength was subtracted from the observed rotation of the protein solution. The specific rotation $[\alpha]$ was then calculated.

Optical rotatory dispersions for paramyosin were plotted according to the Moffitt equation (Moffitt, 1956):

$$[\alpha]_\lambda = \left(\frac{100}{M}\right)\left(\frac{n^2+2}{3}\right)\left[\frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right] \quad (1)$$

where M is the average residue weight and n is the refractive index of the solvent. The value of λ_0 was taken to be 2120 A in accordance with that found best for poly-γ-benzyl-L-glutamate in all solvents (Moffitt and Yang, 1956). In the wavelength range investigated, no deviation from linearity of the Moffitt plot could be detected for several different λ_0 values between 2100 and 2200 A. The parameter b_0 was evaluated from $\frac{M}{100}\left(\frac{3}{n^2+2}\right)/\lambda_0^4$ times the slope of a plot of $[\alpha](\lambda^2-\lambda_0^2)$ vs. $\frac{1}{\lambda^2-\lambda_0^2}$.

When b_0 in equation (1) becomes small, the data can also be plotted according to a one-term Drude equation,

$$[\alpha] = \frac{k}{(\lambda^2-\lambda_c^2)} \quad (2)$$

The parameter λ_c was derived from the slope of the straight line of a modified Drude plot (Yang and Doty, 1957) of $\lambda^2[\alpha]$ *vs.* $[\alpha]$.

Since the concentration of the protein solutions was of the order of 0.6% in KCl and 0.3% in GU, the degree of precision of $[\alpha]$ was ±0.7° and ±1.3° respectively. Consequently, the expected error in b_0 is about ±10°.

Results

Optical Rotatory Properties in 0.3 M KCl.—At pH 7.4 in 0.3 M KCl $[\alpha]_{589}^{20}$ was found to be −14.5°, and the Drude plot (Fig. 1) was found to be anomalous. The Moffitt plot (Fig. 2), however, was linear and yielded a b_0 of −570°.

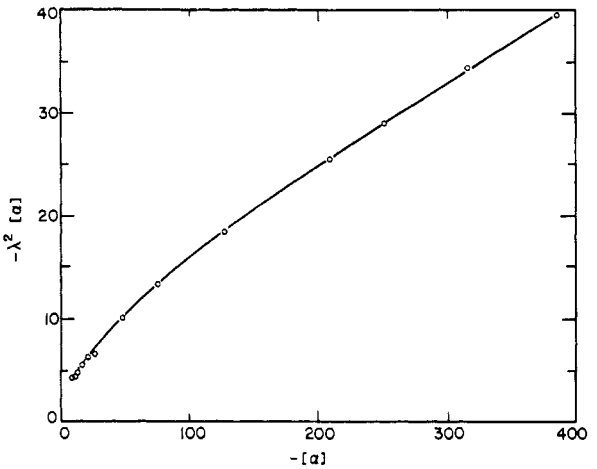

FIG. 1.—A modified Drude plot of the rotatory dispersion of paramyosin in 0.3 M KCl, 0.01 M pH 7.4 phosphate buffer at 20° C. The curvature in the plot is anomalous.

As can be seen in Table I, there was very little pH dependence noted either in specific rotation at 5890 A or in b_0. The slight apparent increase in levorotation with the concomitant decrease in the absolute value of b_0 noted at pH 2.0 may not be real, since the concentration may be inaccurate due to the use of the neutral pH extinction coefficient. Harrap and Doty (1960) found a gradual decrease in extinction coefficient with decreasing pH. However, no values were given, and, in the present study, various means of determining the

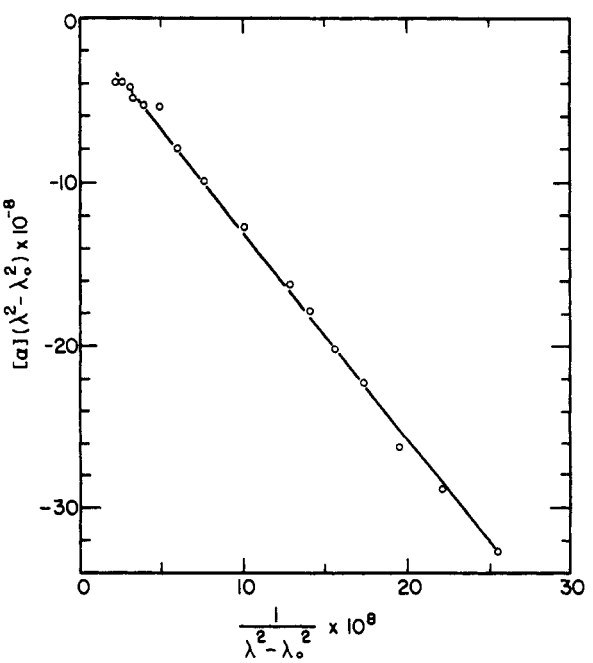

FIG. 2.—A Moffitt plot of the rotatory dispersion of paramyosin in 0.3 M KCl, 0.01 M pH 7.4 phosphate buffer at 20° C.

TABLE I

OPTICAL ROTATORY PROPERTIES OF PARAMYOSIN IN 0.3 M KCl AT 20° C

pH	$[\alpha]_{589}$	b_0
2.0	−18.5°	−511°
7.4	−14.6°	−570°
10.5	−12.5°	−585°

concentration at neutral pH, from which the concentration at pH 2.0 could be accurately derived, were tried without success. A decreased extinction coefficient would make the concentration greater than calculated, thus the absolute magnitude of $[\alpha]_{589}$ less.

In an attempt to follow the denaturation of this molecule, the rotation at 5460 A, with use of the Hanovia mercury lamp, was studied as a function of temperature. However, turbidity developed in a pH 7.0 solution at about 37° C, and no transition had been observed up to that temperature. At pH 2.0 a slight turbidity developed at about the same temperature, but measurements could be continued up to 65° C, with an observed increase of levorotation of about 15°. However, because of the uncertainty in measuring the concentrations at this pH and because the final levorotation (−40°) at 65° C was smaller than the −63° found in 9.5 M urea (Cohen and Szent-Györgyi, 1957), experiments with heating in KCl were abandoned.

Optical Rotatory Properties in GU.—In GU at pH 5.7 $[\alpha]_{589}^{20}$ was found to be about −66°. For comparison purposes, $[\alpha]_s$ may be converted to the corresponding value in water by the formula suggested by Schellman (1958):

$$[\alpha]_w = [\alpha]_s \frac{n_w^2 + 2}{n_s^2 + 2}$$

where n_w and n_s are the indices of refraction of water and the solvent, respectively. When this correction is made, $[\alpha]_{589}^{20}$ becomes −63°. This may be compared to $[\alpha]_{589}^{20}$ of −15° in 0.3 M KCl, indicating an increase in randomness due to the GU solvent.

The Drude plot (Fig. 3) was found to be linear for this solvent and to yield 2440 A for λ_c at 25° C. Moffitt plots were also made of the same data in an attempt to obtain more information about the conformation in this solvent. From Figure 4, a typical Moffitt plot at 25° C, b_0 was found to be about −165°, indicating that approximately one third of the original helical structure remains at room temperature in this denaturing solvent.

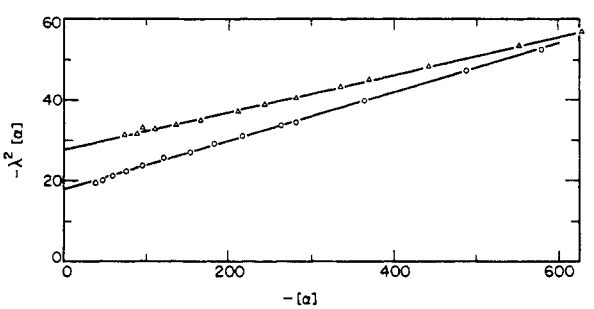

FIG. 3.—Typical modified Drude plots of the rotatory dispersion of paramyosin in GU–0.3 M KCl. The circles represent data for the protein in a pH 5.7 solution at 25° C. The triangles represent data in a pH 3.0 solution at 60° C.

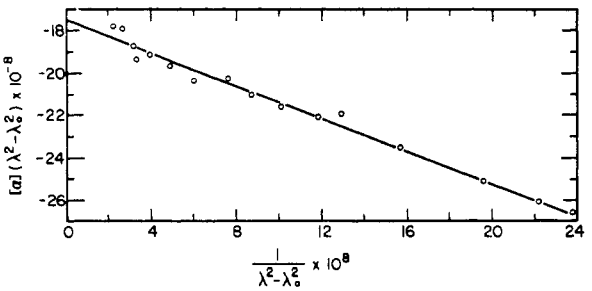

FIG. 4.—A typical Moffitt plot of the rotatory dispersion of paramyosin in GU–0.3 M KCl at 25° C, pH 5.7.

Upon heating of the GU solution, a further increase in levorotation and in b_0 was noted. The data for different pH values are presented in Figures 5–8 in terms of both $[\alpha]_{589}$ and b_0, and the total observed changes (an increase of about 25° in levorotation and of b_0 to about −20°) are found to be pH-independent. However, the transition temperature, t_{tr}, is pH dependent in a manner indicating that the protein in GU is more stable at low rather than at high pH (Fig. 9). t_{tr} is obtained from Figures 5–8 as the temperature at which half the maximum transition is observed, but the curve in Figure 9 is drawn through the points derived from the $[\alpha]_{589}$ *vs.* temperature curves (Fig. 5 and 6), since these curves are better defined in the transition region. A comparison of typical Moffitt plots for pH 3.0 and pH 5.7 solutions at 40° C in Figure 10 shows that greater unfolding has occurred in the pH 5.7 solution at this temperature. In a plot of b_0 *vs.* pH at different temperatures (Fig. 11), the pH dependence of t_{tr} is illustrated by curve 3 for 40° C. It can also be seen that there is no pH dependence at either lower or higher temperatures.

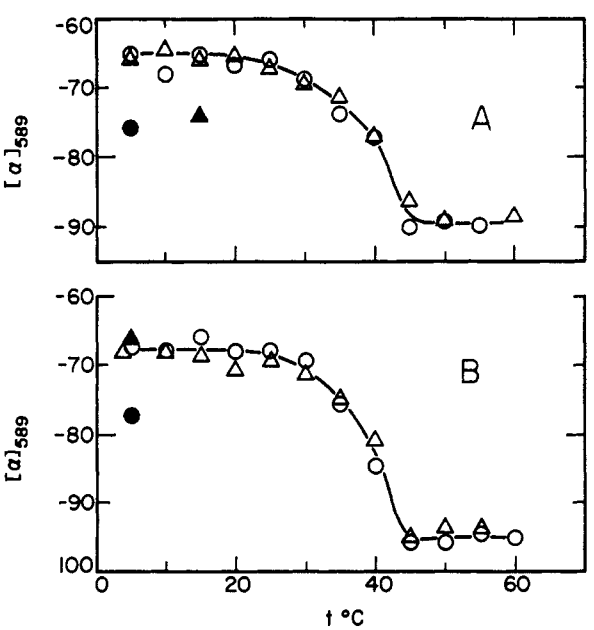

FIG. 5.—Optical rotation of paramyosin in GU–0.3 M KCl as a function of temperature. The triangles and circles represent two separate experiments, and their size indicates the expected error. The filled symbols represent data taken after cooling the solution for at least 5 hours. *A*, pH 2.0. *B*, pH 3.0.

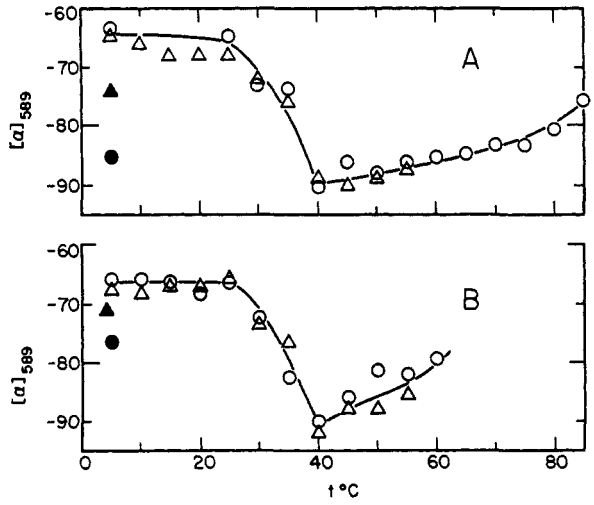

FIG. 6.—Optical rotation of paramyosin in GU–0.3 M KCl as a function of temperature. The symbols have the same meaning as in Figure 5. *A*, pH 5.7. *B*, pH 10.2.

If the maximum temperature is kept below 60° C, this heat-induced transition is essentially reversible (as indicated in Fig. 5–8 by the solid symbols) to the value noted at room temperature, but rarely

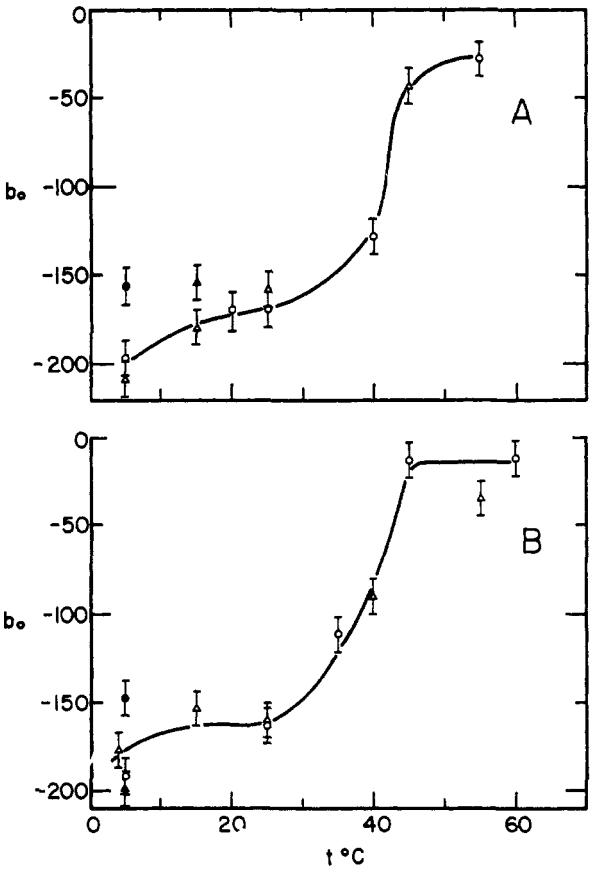

FIG. 7.—The Moffitt parameter b_0 for paramyosin in GU–0.3 M KCl as a function of temperature. b_0 was derived from plots such as the one in Figure 4. The circles and triangles denote different experiments, and the expected error is indicated. The filled symbols indicate dispersion measurements made after the solution had been cooled for at least 5 hours. *A*, pH 2.0. *B*, pH 3.0.

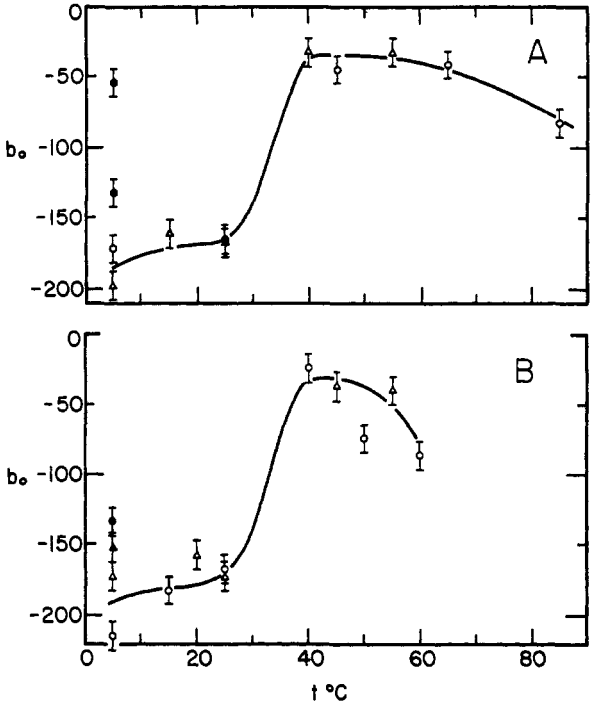

FIG. 8.—The Moffitt parameter b_0 for paramyosin in GU–0.3 M KCl as a function of temperature. The symbols have the same meaning as in Figure 7. *A*, pH 5.7. *B*, pH 10.2.

to the original value at 5° C.

The negative temperature coefficient of levorotation observed in pH 5.7 and pH 10.2 solutions above 50° C (Fig. 6) is characteristic of a random coil configuration (Kauzmann and Eyring, 1941; Schellman and Schellman, 1958). Therefore, the increased absolute value of b_0 probably is a function of this temperature coefficient of the random coil and does not indicate a refolding of the molecule.

A typical Drude plot at high temperature is compared to one at 25° C in Figure 3. The value for λ_c is found to be 2150 A at 60° C for pH 3.0 solution.

DISCUSSION

Conformation of the Native Protein.—The Moffitt parameter b_0 should be regarded only as empirical,

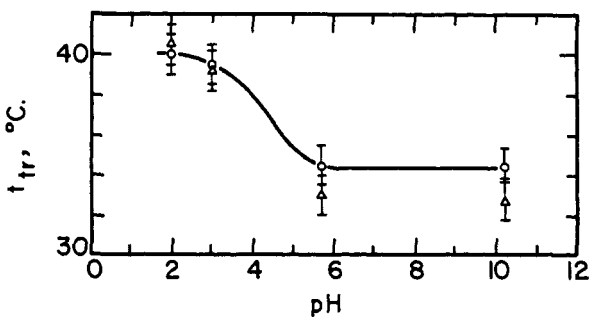

FIG. 9.—The transition temperature as a function of pH. The circles represent values obtained from $[\alpha]_{589}$ *vs.* temperature data (Fig. 5 and 6), and the triangles represent values obtained from b_0 *vs.* temperature data (Fig. 7 and 8). Expected error is indicated. The curve is drawn through the circles, as they represent data better defined in the transition region.

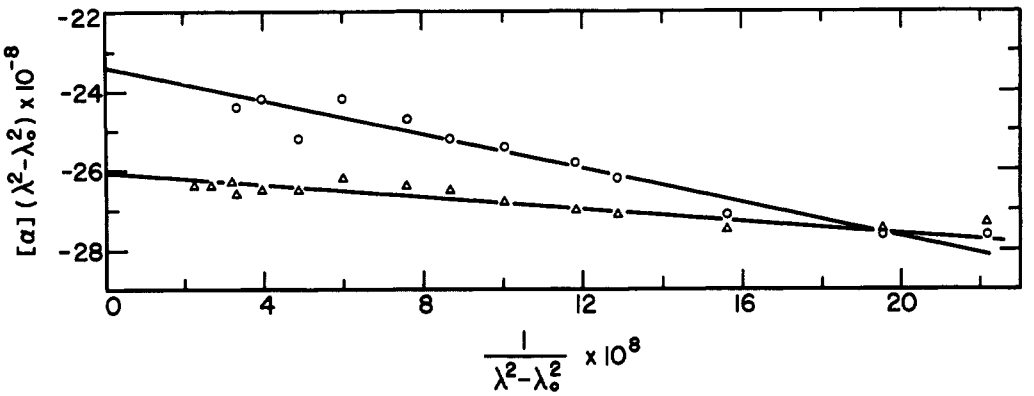

FIG. 10.—Moffitt plots for paramyosin in GU–0.3 M KCl at 40° C. The circles indicate a pH 3.0 solution and the triangles a pH 5.7 solution.

since certain features of Moffitt's theoretical evaluation are now known to be incorrect (Moffitt *et al.*, 1957). Yet b_0 can serve as a guide to changes in helical content for a particular protein. For paramyosin in KCl, b_0 is so close to the value −600° characteristic of a bare right-handed α-helix for λ_0 of 2120 A (Imahori, 1960) that the native molecule must exist mainly in this form, as has been previously concluded by Cohen and Szent-Györgyi (1957), Kay and Bailey (1959), and Harrap and Doty (1960). The anomalous Drude plot is due to this high helical content. The high degree of helicity of the molecule is not surprising, since this preparation contains only nine prolines per 2800 residues (Riddiford and Scheraga, 1962). Proline content has been inversely correlated with high helical content by Szent-Györgyi and Cohen (1957).

Conformation in GU at Low Temperature.—The linearization of the Drude plot, the decreased absolute value of b_0, and the increased levorotation to −63° (corrected to water) when native paramyosin is put into GU all indicate unfolding of the molecule. However, as discussed by Schellman and Schellman (1961), a λ_c above 2300 A rules out a random polypeptide chain configuration. λ_c of 2440 A thus suggests helical structure, and b_0 of −165° for paramyosin in pH 5.7 GU at 25° C indicates that approximately one third of the helical structure is retained in this solvent. Also, b_0 and $[\alpha]_{589}$ in GU agree well with values found by Cohen and Szent-Györgyi (1957) for *Venus* paramyosin in 9.5 M urea. The first stage of unfolding evidently involves the regions of the molecule in which tyrosyl interactions occur, because in GU, all the tyrosyl groups can be titrated at 25° C (Riddiford and Scheraga, 1962). Since the partially unfolded molecule probably consists of random regions interspersed with helical regions, the apparent rodlike character in GU (Riddiford and Scheraga, 1962) may be maintained by intermolecular interactions involved in the coiled coil conformation (Cohen, 1961). This coiled coil conformation may be responsible for the observed difference in denaturation properties between *Pinna* tropomyosin in 8 M urea (completely reversibly unfolded, Kay and

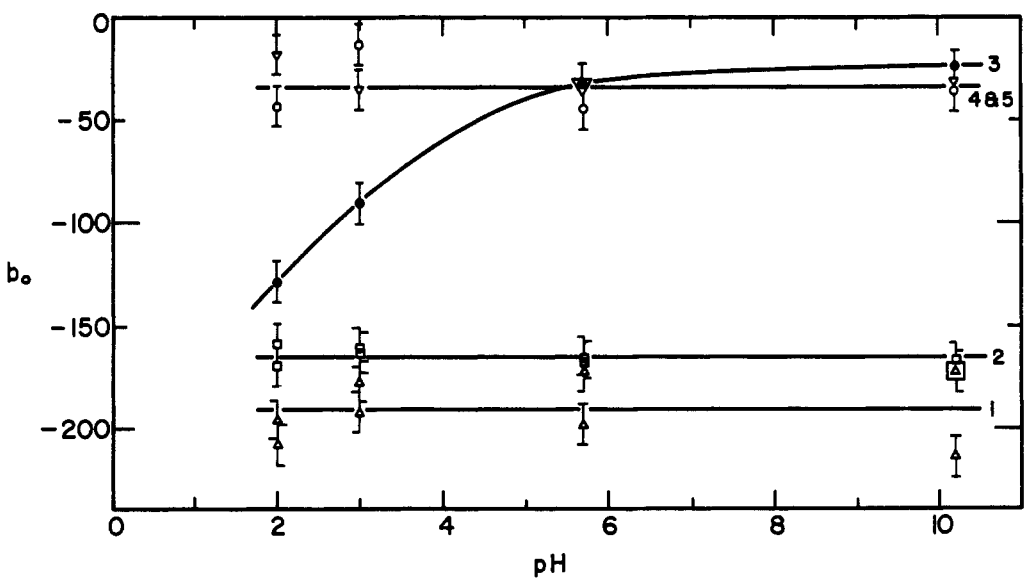

FIG. 11.—The Moffitt parameter b_0 for paramyosin in GU–0.3 M KCl as a function of pH at five temperatures. Expected error is indicated. 1. △ = 5° C. 2. □ = 25° C. 3. ● = 40° C. 4. ○ = 45° C. 5. ▽ = 55° C.

Bailey, 1959) and *Venus* paramyosin in 9.5 M urea (partially denatured to the same extent as in GU, Cohen and Szent-Györgyi, 1957), because *Pinna* tropomyosin is presumably a single α-helical chain (Kay, 1958) and therefore would be expected to be less stable than a coiled coil. Although no attempt was made to demonstrate the reversibility of this first-stage transition in GU, it may be reversible, since the complete denaturation of *Pinna* tropomyosin is claimed to be reversible (Kay and Bailey, 1959).

Conformation in GU at High Temperature.—The development of turbidity upon heating of paramyosin in KCl has been noted previously by Harrap and Doty (1960) and suggested to them the possible formation of a β-structure. However, they found no infrared evidence for a β-structure under these conditions and concluded that a more likely structure was a solvated coil arrangement with multiple intermolecular noncovalent cross-links. Therefore, the absence of any turbidity upon prolonged heating in GU probably can be attributed to the disruption of these interactions by GU.

The first stage of unfolding seems to occur quickly —within the time required for measurement. The second stage of unfolding is also rapid, and appears to depend on temperature; this permits a study of the second-stage unfolding process. By the criteria of $\lambda_c \cong 2150$ A, $b_0 = -10°$ to $-30°$, and $[\alpha]_{589} \cong -90°$, complete transition to the random polypeptide form seems to have occurred upon heating to 50° C. Paramyosin at high temperature could have one of three possible conformations on the basis of only $\lambda_o \cong 2150$ A (Schellman and Schellman, 1961): a random coil, a β-structure, or a left-handed helix. However, the b_0 and the $[\alpha]_{589}$ values are in better agreement with the values expected for a random coil (Doty, 1958; Schellman and Schellman, 1961) than for the other two conformations.

Reversibility of Unfolding in GU.—The essential reversibility of this second-stage transition was demonstrated for temperatures below 60° C. However, irreversible changes occur above 60° C. Heating of aqueous urea solution is known to accelerate the decomposition of urea to ammonium cyanate, and cyanate has been found to react with ϵ-amino and sulfhydryl groups in proteins (Stark *et al.*, 1960). A significant increase in alkalinity of GU solutions heated to 60° C or higher for about an hour, then cooled, indicated the presence of ammonia. The interaction of the cyanate so formed with the unfolded paramyosin could prevent refolding if either the ϵ-amino or sulfhydryl groups were involved in maintaining the partially helical molecule at 25° C.

pH-dependence of the Second-stage Transition.—The observed pH dependence of the transition temperature, t_{tr}, may provide an explanation for the two-stage unfolding of *Venus* paramyosin. Although the difference in t_{tr} is only 6° C (Fig 9), the striking dependence of b_0 upon pH only at 40° C (Fig. 10 and 11) confirms its significance. It is difficult to understand this low pH stability in GU. One possibility is the presence of carboxyl-carboxyl hydrogen bonds (Scheraga, 1960). In such a case, a Δt_{tr} of 6° would correspond to one carboxyl-carboxyl bond per 50 residues, based on the same parameters used for calculations by Scheraga (1960), assuming the absence of cross-links. If a molecular weight of 330,000 were to correspond to a three-stranded coil, there would be eighteen carboxyl-carboxyl bonds per chain. However, such bonds were not detectable by titration in either KCl or GU (Riddiford and Scheraga, 1962). Perhaps molecular rearrangements occurring within the coiled coil conformation during the first-stage unfolding bring carboxyl groups into favorable positions (possibly in hydrophobic regions) so that these bonds can form. The peculiarity noted in the carboxyl ionization in GU below pH 3.0 (Riddiford and Scheraga, 1962) probably prevents the detection of these bonds by titration.

The absence of hydrogen bonds detectable by titration (Riddiford and Scheraga, 1962) in the first-stage unfolding and, at most, only eighteen hydrogen bonds per chain in the second-stage unfolding, led us to conclude that most of the nonelectrostatic stabilization of the native conformation (including that of the coiled coil) arises from interactions between nonpolar groups. Because the second-stage unfolding was found to be irreversible above 60° C, at which temperature urea decomposes, sulfhydryl and ϵ-amino groups may also be involved in stabilizing interactions.

Since paramyosin constitutes a major portion of the molluscan muscles responsible for prolonged contraction (Szent-Györgyi, 1960), the intrinsic stability of this protein in solution would be of great importance in its physiologic role. The intermolecular interactions, which apparently occur in solution as a function of pH and ionic strength (Riddiford and Scheraga, 1962) and which appear to increase molecular stability, must be involved in this sustained contraction, no matter which of the several proposed physiologic mechanisms (Prosser, 1960) is involved.

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Anomalous Rotatory Dispersion of Enzyme Complexes. III. Rotatory Dispersion Titration of Liver Alcohol Dehydrogenase with Coenzyme Analogues and *p*-Chloromercuribenzoate*

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The stoichiometry of the interaction of the analogues of DPNH with liver alcohol dehydrogenase has been measured by means of *rotatory dispersion titration*. At pH 7.5 to 9.5—as with DPNH—2 moles of desamino DPNH, 3-acetylpyridine DPNH, and thionicotinamide DPNH bind asymmetrically to each mole of liver alcohol dehydrogenase. Between pH 9.5 and 11, the liver alcohol dehydrogenase–DPNH complex progressively dissociates; the midpoint of the curve is at about pH 10. *p*-Chloromercuribenzoate also dissociates the enzyme-coenzyme complex; a twentyfold molar excess of this agent prevents completely the binding of DPNH and its analogues to liver alcohol dehydrogenase. However, simultaneously, the enzyme undergoes changes in optical rotation indicative of protein denaturation; hence the effect of *p*-chloromercuribenzoate on coenzyme binding and catalytic activity cannot be attributed solely to the interaction of this agent with a specific sulfhydryl group or groups at the active enzymatic site.

Measurement of the binding of coenzymes, substrates, and inhibitors to enzymes is an important experimental problem in elucidating the mechanisms of enzyme action and in ascertaining the nature of active enzymatic sites. In this regard, both the chemistry of the interacting groups of these molecules and their arrangement in three-dimensional space are critical. Thus far, physicochemical methods which jointly examine both of these factors have been lacking (Fraenkel-Conrat, 1960; Koshland, 1960).

Recently we have reported that spectropolarimetry can be employed to study directly the composition, structure, and function of active centers of certain enzymes; the binding of chromophoric molecules at these asymmetric sites induces anomalous optical rotatory dispersion, a Cotton effect, in the absorption bands of the bound chromophores (Ulmer and Vallee, 1961). By means of such Cotton effects, asymmetric enzyme-metal-chelate complexes have been identified qualitatively, and the interactions of coenzymes and inhibitors at the active centers of enzymes have been characterized (Ulmer *et al.*, 1961a). Moreover, it has been learned that the magnitude of such Cotton effects can be used to determine binding quantitatively.

The present work extends previous observations and describes the quantitative measurement of the interactions of liver alcohol dehydrogenase with coenzyme analogues, and the inhibitor, *p*-chloromercuribenzoate, by a procedure which we have termed *rotatory dispersion titration*. The binding to liver alcohol dehydrogenase of each of the coenzyme analogues is identified by the presence of a characteristic Cotton effect centered at the absorption maximum of the enzyme-analogue complex (Ulmer *et al.*, 1961b). The magnitude of the Cotton effect is dependent upon the concentration of this complex, which is, thereby, titrated by rotatory dispersion, but it is independent of the concentration of free analogue and apoenzyme.

MATERIALS AND METHODS

Crystalline alcohol dehydrogenase of horse liver was obtained from C. F. Boehringer and Soehne, Mannheim, West Germany. Prior to use, the enzyme was dialyzed for five days against 0.1 M sodium phosphate buffer, pH 7.5, at 4°, to remove low-molecular-weight impurities which absorb radiation at 280 mμ. In the same buffer at pH 7.5 the enzyme was monodisperse upon sedimentation in the analytical ultracentrifuge (Spinco, Model E). The concentration of protein was determined by

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