Unfolding Reactions of Proteins. II. Spectral and Optical Rotatory Dispersion Studies in Urea, Guanidinium Chloride, and 2-Chloroethanol*

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ABSTRACT: The denaturation of α-chymotrypsin and diisopropylphosphorylchymotrypsin in urea and guanidinium chloride has been examined by difference absorbance and optical rotatory dispersion measurements. In either denaturant, both proteins have a maximal blue shift (corrected for solvent effects) corresponding to a molar difference extinction coefficient of −10,000 at 293 mμ. In the denatured state, the a_0 dispersion parameter is large and negative, whereas b_0 is approximately zero. The temperature coefficient for optical rotation is negative. Thermodynamically and kinetically, all changes in absorbance and optical rotation occur in phase and it appears that a single cooperative unit participates in the transition from the native to the denatured state. In contrast, and on the basis of (a) kinetic and equilibrium measurements of spectral and optical rotation changes, (b) temperature transition studies, and (c) comparison with the behavior of model compounds, solution of diisopropylphosphorylchymotrypsin into high concentrations of 2-chloroethanol results in a structural ordering reaction with a partially denatured state as an intermediate. Insofar as changes in absorbance and optical rotation reflect the events that occur, the process takes place in sequential fashion. The spectral changes are essentially complete before the optical rotation change (composed of a fast and slow reaction) to a less levorotatory value has begun. In the transition from native to partially denatured to the ordered state, it seems that at least three cooperative units are involved.

Compared to the relatively large amount of available literature concerned with the denaturation of proteins by such agents as urea, guanidinium chloride, and detergents, a paucity of data exists for the effect of organic solvents on the physical properties of proteins. Optical rotatory dispersion (ORD) behavior of a number of proteins in chloroethanol has been the subject of study by Doty and colleagues (Urnes and Doty, 1961), Shechter and Blout (1964), Herskovits and Mescanti (1965), and Herskovits (1966). The effect of chloroethanol on ribonuclease (Weber and Tanford, 1959) and muramidase (Hamaguchi and Kurono, 1963a) has also been investigated. By analogy with the results obtained for the behavior of polyamino acids in this alcohol and other "helix-forming" solvents (Urnes and Doty, 1961; Singer, 1962), chloroethanol has been considered to promote a greater degree of helical content in proteins. From the results of equilibrium measurements, it has also been suggested that the path of a protein from the native state in water to that in chloroethanol may involve a structurally disorganized intermediate(s) (Weber and Tanford, 1959; Hamaguchi and Kurono, 1963a). A similar conclusion has also been reached by Bigelow and Krenitsky (1964) from a study of the spectral changes of ribonuclease in dioxane–water solutions.

Chloroethanol, among other solvents, has been studied by Herskovits (1965) as to its ability to promote exposure of the phenol and indole chromophores of proteins. In general, the extent of exposure was enhanced and about the same as that obtained in 8 M urea although ORD measurements (Herskovits and Mescanti, 1965) showed a marked rise in the Moffitt b_0 dispersion parameter to more negative values.

In a continuation of our studies concerned with kinetic and equilibrium measurements of the physical properties of chymotrypsin and its derivatives upon denaturation (Martin, 1964; Martin and Bhatnagar, 1965, 1966), we have examined the effect of urea and guanidinium chloride on the spectral changes and optical rotatory behavior of CT[1] and DIPCT. The effect of 2-chloroethanol on the latter protein has also been investigated. Both urea and guanidinium chloride produce essentially identical denatured states. In contrast, in high concentrations of chloroethanol, and on the basis of both equilibrium and rate measurements, the transconformation reaction of DIPCT proceeds through a transitory partially denatured

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[1] Abbreviations used: CT, α-chymotrypsin; DIPCT, diisopropylphosphorylchymotrypsin.

intermediate before assuming a more ordered structure.

Experimental Procedure

The CT preparations (three-times-crystallized, salt-free, Lot No. CDI-6093 and CDI-6096-7) and muramidase (two-times-crystallized, salt-free, Lot No. LYSF-632 and a preparation containing salt, Lot No. LY-633, but dialyzed before use) were obtained from Worthington Biochemical Corp. The DIP derivative of CT was made as previously described (Martin and Bhatnagar, 1966). Urea and guanidinium chloride were reagent grade materials and were purified as before (Martin and Frazier, 1963; Martin, 1964). The 2-chloroethanol was an Eastman product and after treatment with potassium carbonate was purified by vacuum distillation using a Vigreaux column. This was done three times, collecting the middle one-third fraction (bp 24° at 1.5 mm). The final material was stored at −15°. Twofold dilution of various samples with water gave an apparent pH within the range of 3.0–3.5. The cutoff point (absorbance equal to 1.00 with 10-mm light path) was at 220 mμ but a small amount of material with selective absorbance at 260 mμ was present. Immediately before use, the 2-chloroethanol was diluted with water to give a 90% solution at the apparent pH of 4.0.

All solutions, with the exception of those containing 2-chloroethanol, were filtered through a Millipore filter (pore size, 0.45 μ) prior to use. An enzymic digest of DIP-CT (treatment first with pepsin and then trypsin) was prepared as described previously (Martin, 1964).

Difference spectral measurements were obtained with the Cary Model 14 recording spectrophotometer and procedural techniques were as previously employed (Martin and Bhatnagar, 1966). All discussions of difference spectra refer to measurements wherein sample in denaturant or perturbant (in sample beam) was compared with sample in water (in reference beam).

ORD measurements were obtained with a Zeiss 0.005° polarimeter at the nominal wavelengths of 578, 546, 436, 405, and 365 mμ. The data were analyzed using the phenomenological equation of Moffitt and Yang (1956) transformed into the form

$$[m']_\lambda \frac{\lambda^2 - \lambda^2_0}{\lambda^2_0} = a_0 + b_0 \frac{\lambda^2_0}{\lambda^2 - \lambda^2_0}$$

for the graphical solution of a_0 and b_0. For the calculation of $[m']_\lambda$, the reduced mean residue rotation, a value of 104 was used for either CT or DIPCT (Havsteen and Hess, 1963). In empirical conformity with present usage, λ_0 was taken as 212 mμ. Specific rotations were corrected for the refractive index of the solvent to that which would obtain in water by the relation (Schellman, 1958a), $[\alpha]'_\lambda = r_s[\alpha]_\lambda$, where $r_s = (n_w^2 + 2)/(n_s^2 + 2)$, n_w and n_s are the refractive indices of water and solvent, respectively, and $[\alpha]_\lambda$ is the specific rotation at wavelength λ. Refractive indices were measured with a Bausch and Lomb Model 3-L Abbe refractometer at 589 mμ and were not corrected for dispersion. All rotation measurements were made in a thermostated 10-cm tube fitted with removable end plates. The protein concentration was between 4 and 5 mg/ml.

All measurements, except those involving temperature transitions, were made at 30.0° and the apparent pH of 4.0. Buffers were not employed. A Radiometer Model TTT1 or 22 meter, standardized against a pH 4.01 (at 30°) phthalate buffer prepared according to National Bureau of Standards specifications, was used for the determination of pH. A G-202B glass and K4312 calomel electrode was used.

The molecular weight of CT and DIPCT was taken as 25,000 and protein concentration was determined as before (Martin, 1964). A molecular weight of 14,400 was used for muramidase (Sophianopoulos *et al.*, 1962). A molar extinction coefficient of 38,740 at 280 mμ was used for the calculation of muramidase concentration (Hamaguchi and Kurono, 1963b).

Viscosity measurements were made in a Cannon-Ubbelohde semi-microdilution viscometer which had a flow time with water of 421.2 sec at 30.0°. Five repetitive flow times were measured on each sample or solvent solution and the average values used for the calculation of the reduced viscosity (η_{red}, in deciliters per gram), defined as

$$\eta_{red} = \frac{1}{c}\left(\frac{\eta - \eta_0}{\eta_0}\right) = \frac{1}{c}\left(\frac{t}{t_0} - 1\right)$$

where t is the average flow time of the sample, t_0 the average flow time of the solvent, and c the protein concentration in g/100 ml. The maximum deviation of any single flow time from the average value was 0.28%.

Results

The Effect of Urea and Guanidinium Chloride

Difference Spectra. The difference spectrum of either CT or DIPCT in high concentrations of urea is characterized by maxima at 293, 285.5, and 231 mμ, a shoulder at 277 mμ, and a broad envelope of positive absorbance from about 240 to 265 mμ (Martin, 1964; Martin and Bhatnagar, 1966). A similar difference spectrum was obtained in 6 M guanidinium chloride.[2] However, the spectral intensity of the peak positions was considerably less than in 8 M urea. Since it seemed improbable that guanidinium chloride was a less effective denaturant than urea (see Gordon and Jencks, 1963), the equilibrium spectral changes of CT and

[2] A previous sample of recrystallized guanidinium chloride was reported (Martin, 1964) to have such high absorption below 270 mμ as to make difference spectral measurements below this wavelength impossible even with 2-mm cuvets. The sample used in the present study and obtained from a different supplier presented no such difficulties.

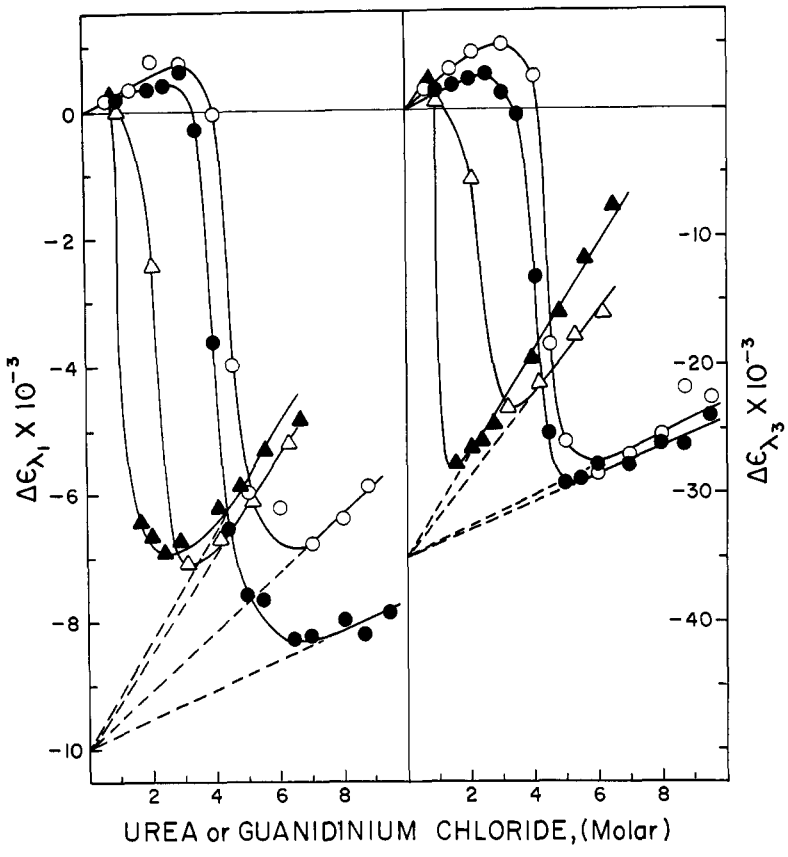

FIGURE 1: Variation in molar difference extinction coefficients at 293 (λ_1) and 231 mμ (λ_3) with denaturant concentration. Open symbols, DIPCT; solid symbols, CT; circles, in urea; triangles, in guanidinium chloride.

DIPCT have been investigated at various concentrations of both denaturing agents.

At low denaturant concentrations, red-shift spectra were observed which, as the concentration was further increased, were replaced by blue-shift spectral effects as both proteins became denatured (Figure 1). The latter were partially reversed to more positive values at still higher urea or guanidinium chloride concentrations. It will be noticed that there is a considerable difference in the slopes for CT and DIPCT in high urea concentrations in the region of tryptophan absorption at 290 mμ. Since the slopes for the two proteins are essentially equivalent in the 230-mμ region, wherein tryptophan also exhibits appreciable absorption (Martin and Bhatnagar, 1966), the reason for this difference is not clear. The points at high concentration of denaturant could be fitted to lines meeting at a common ordinate intercept for a given wavelength. The intercept values at zero denaturant concentrations define the molar difference extinction coefficients ($\Delta\epsilon_M$) for denaturation corrected for solvent effects (Bigelow, 1964; Martin and Bhatnagar, 1966). The values, $-10{,}000$ at 293 mμ and $-35{,}000$ at 231 mμ, for the denaturation of both proteins in either urea or guanidinium chloride are identical with those obtained for the denaturation of DIPCT in urea containing 0.20 M $CaCl_2$ (Martin and Bhatnagar, 1966).

It has been previously shown, in conformity with the results of Williams *et al.* (1965), that about 3 tryptophyl equiv out of 7 (Wilcox *et al.*, 1957) in native DIPCT are exposed to the solution environ-

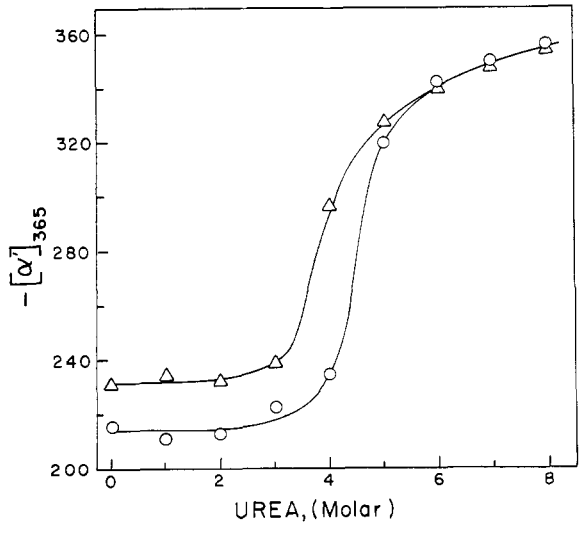

FIGURE 2: The change in optical rotation with urea concentration. (Δ) CT; (O) DIPCT.

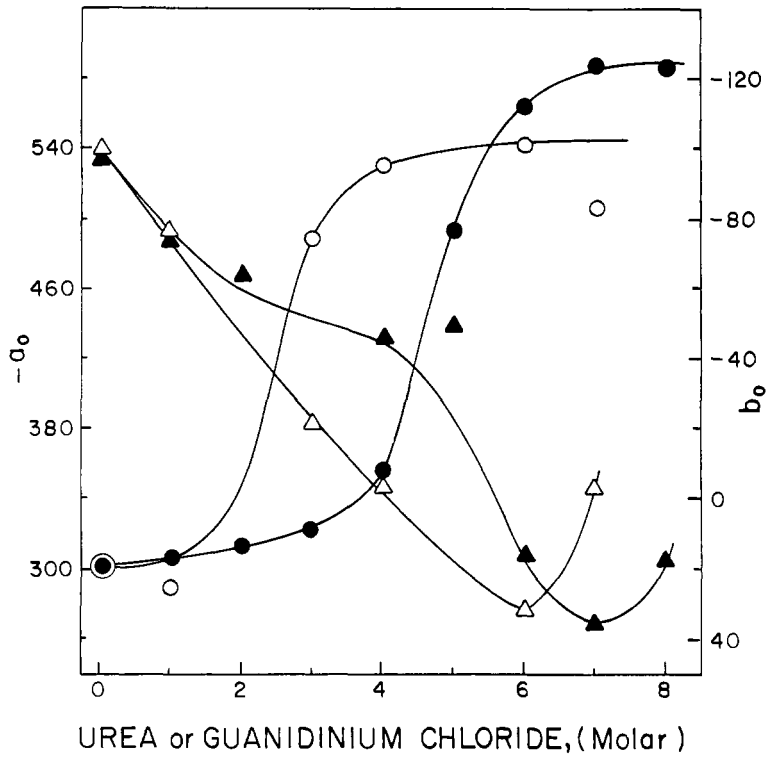

FIGURE 3: Change in the Moffitt dispersion parameters of DIPCT with denaturant concentration. Circles, change in a_0; triangles, change in b_0; open symbols, in guanidinium chloride; solid symbols, in urea.

ment at pH 4.0 (Martin and Bhatnagar, 1966). This was based on a comparison of the initial slope of $\Delta\epsilon_M$ *vs.* denaturant (urea in 0.20 M $CaCl_2$) concentration plot and red-shift spectral data for model compounds in the region of tryptophan absorption. By analogous procedures, one can determine from data obtained in urea solution (Figure 1) that 3.3 and 2.6 tryptophyl equiv are exposed in the native state for DIPCT and CT, respectively. The difference between the two proteins is not considered significant. In fact, other evidence (Oppenheimer *et al.*, 1963; Williams *et al.*, 1965) suggests that the tryptophyls are slightly less accessible in DIPCT than in CT. From the above results, *i.e.*, three tryptophyls exposed, and the value of the ordinate intercept for $\Delta\epsilon_M$ at 293 mμ, the transfer of 1 tryptophyl equiv from the "interior" of the protein to the aqueous environment is attended with a value for $-\Delta\epsilon_M$ of 10,000/4 or 2500. This agrees with previous measurements (Martin and Bhatnagar, 1966).

The results of Figure 1 also demonstrate the greater effectiveness of guanidinium chloride as a denaturant compared to urea. For example, one-half the total spectral change of CT at either wavelength is achieved in about 1.0 M guanidinium chloride; a urea concentration of about 4.0 M is required for the same effect. The equivalent change for DIPCT required about 2.2 M guanidinium chloride or 4.5 M urea. If one were to compare the spectral changes elicited by both denaturants at only a single high concentration of each, an opposite conclusion would be reached. The results also show that in either denaturant, DIPCT is thermodynamically more stable than CT and that the changes

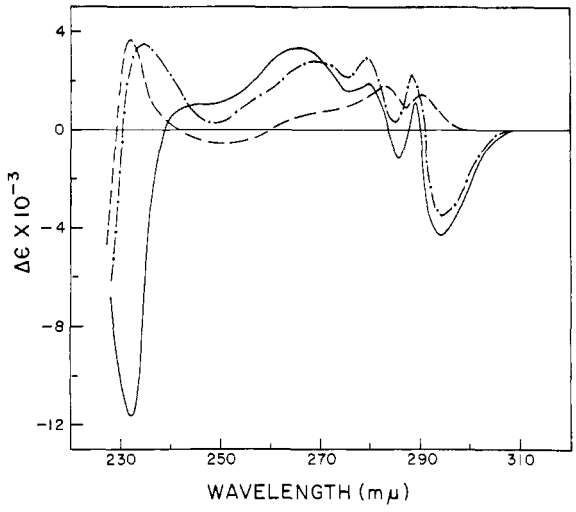

FIGURE 4: Difference spectra of DIPCT in chloroethanol–water solutions *vs.* DIPCT in water. The chloroethanol concentration was 10 (- - - -), 40 (———), and 80 (- · - - ·) vol. %.

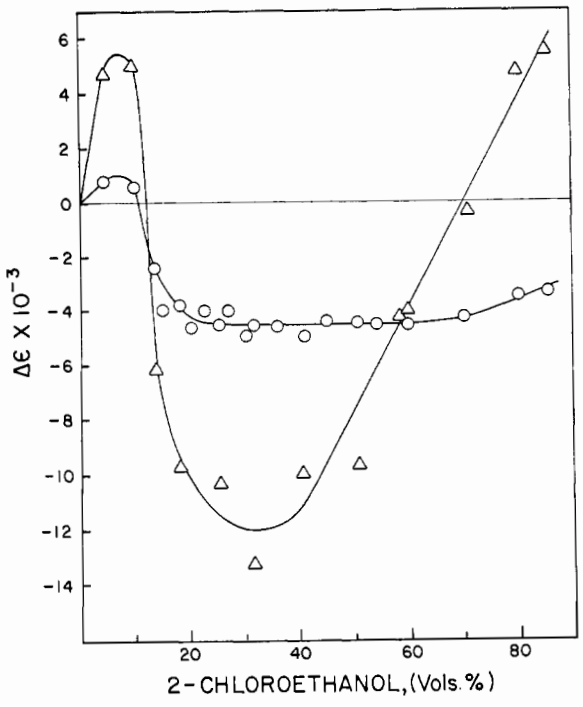

FIGURE 5: Variation in the molar difference extinction coefficient of DIPCT with chloroethanol concentration. (O) at 292–296 mμ; (Δ) at 231–235 mμ.

at 293 and 231 mμ occur in phase. From a kinetic standpoint, DIPCT is also more stable than CT (Martin and Bhatnagar, 1966).

It has been previously shown that the presence of calcium ions exerts a protective effect on the rate of denaturation of both CT (Martin, 1964) and DIPCT (Martin and Bhatnagar, 1966). Below a pH of 4.5, the rate of denaturation was the same in the absence as in the presence of this cation. It was suggested that calcium ions stabilized the native conformation of both proteins at higher pH values by possible binding to two anionic sites. It would be expected then that the concentration of urea to produce the half-unfolded state (by the criterion of spectral change) at pH 4.0 would be independent of the presence of $CaCl_2$. As mentioned above, this state was attained for DIPCT at a urea concentration of 4.5 M. This agrees well with the value obtained (4.4 M) in the presence of 0.20 M $CaCl_2$ (Martin and Bhatnagar, 1966).

Optical Rotation Studies. In agreement with spectral data, a higher urea concentration is required to produce a change in the optical rotation of DIPCT comparable to that of CT (Figure 2). For CT, the midpoint of the optical rotation transition occurred at about 3.8–4.0 M urea; for DIPCT, the same point was attained at 4.5 M. The denaturation reaction thus proceeds with optical rotation and spectral changes coincidental.

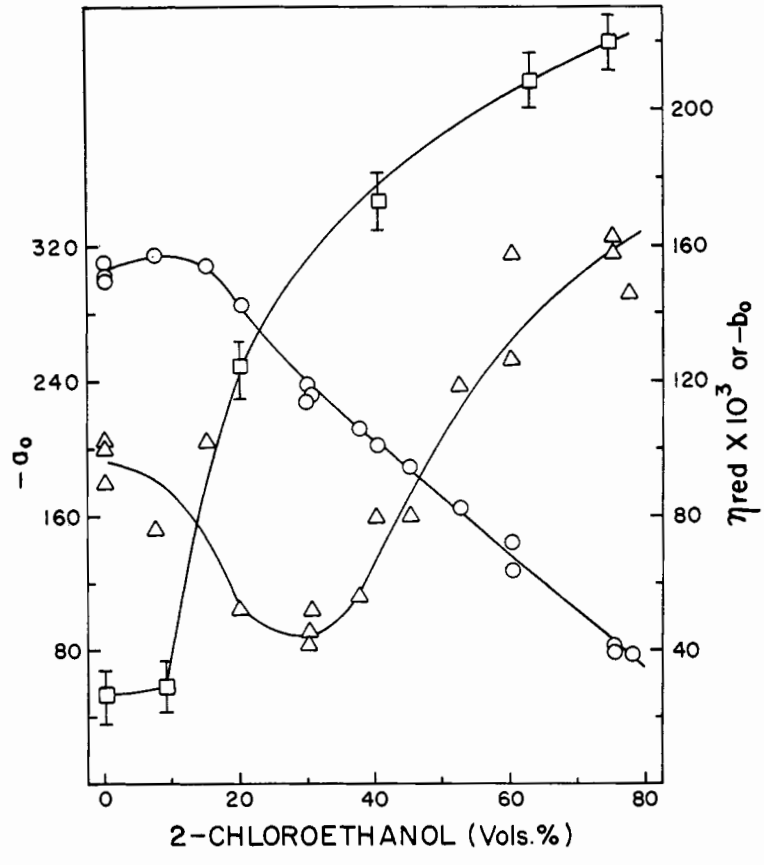

FIGURE 6: Change in the Moffitt dispersion parameters and reduced viscosity of DIPCT with chloroethanol concentration. (O) change in a_0; (Δ) change in b_0; (□) change in reduced viscosity (0.493 g/100 ml).

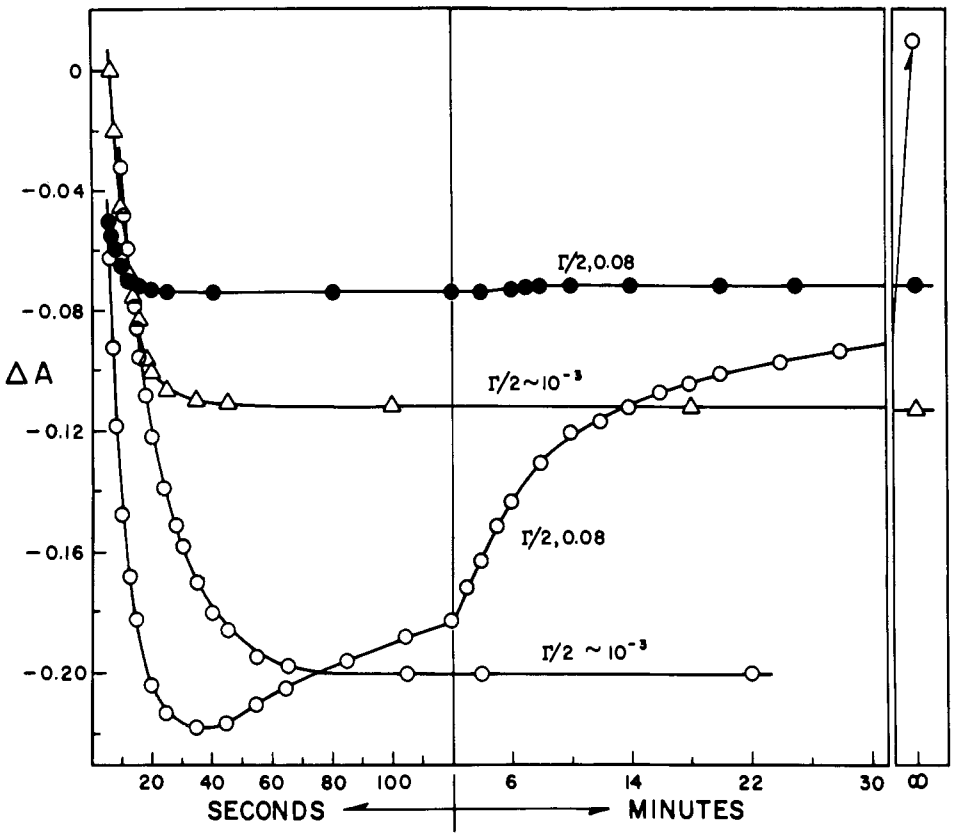

FIGURE 7: The rate of change in difference absorbance of DIPCT in chloroethanol at indicated ionic strengths. Open symbols, at 231 mμ; solid symbol, at 293 mμ; circles, in 30 vol. %; triangles, in 40 vol. %.

Rate studies of the denaturation process have also shown that the changes in these parameters, in their approach to equilibrium values, occur synchronously (Martin, 1964; Martin and Bhatnagar, 1966).

ORD measurements of DIPCT in urea and guanidinium chloride fitted the Moffitt–Yang expression and showed that the a_0 dispersion parameter increased to -540 or -590 and that b_0 decreased to about zero as the denaturant concentration was increased (Figure 3). The change in optical rotation (reflected in the a_0 dispersion parameter) in guanidinium chloride occurred in phase with the spectral change shown in Figure 1.

The Effect of 2-Chloroethanol

All measurements in chloroethanol–water solutions have been made with DIPCT to avoid possible complications of autolysis. In the initial experiments concerned with the spectral changes of DIPCT in chloroethanol, it was observed that final equilibrium values in the 230-mμ region were attained only after about 24-hr incubation. This contrasted with the relatively rapid attainment of final values in the 290-mμ region. This behavior was traced to an ionic strength effect and due to inadequate removal of HCl from the chloroethanol. All experiments herein reported, therefore, with a single exception (see below), have been done at an ionic strength of about 0.001 using chloroethanol purified as described in the Experimental Section.

Difference Spectra. At 10 vol. % chloroethanol, a red-shift spectrum was obtained with peaks of positive absorbance at 290.5, 283, and 232 mμ (Figure 4). At 40 vol. %, a denaturation spectrum similar to that seen in urea or guanidinium chloride appeared with maxima at 293.5, 285.5, and 232 mμ. The spectrum became anomalous at 80 vol. % chloroethanol. The low-wavelength peak was now positive and shifted to the red end of the spectrum (to 234.5 mμ). A slight shift (to 294.5 mμ) also occurred in the region of tryptophan absorption, but the decrease in peak intensity was much less.

Final equilibrium values for $\Delta\epsilon_M$ due to tryptophan absorption and in the 230–235-mμ spectral region in various chloroethanol–water solutions are presented in Figure 5. Surprisingly, the magnitude of the tryptophan difference peak remained constant from about 20 to 70 vol. % chloroethanol.

ORD Measurements. The optical rotation of DIPCT fitted the Moffitt–Yang equation over the wavelength range available (578–365 mμ). The dispersion parameters remained essentially unchanged as the alcohol concentration was increased to 15 vol. % (Figure 6). Above this concentration, both quantities became more positive. However, above 30 vol. %

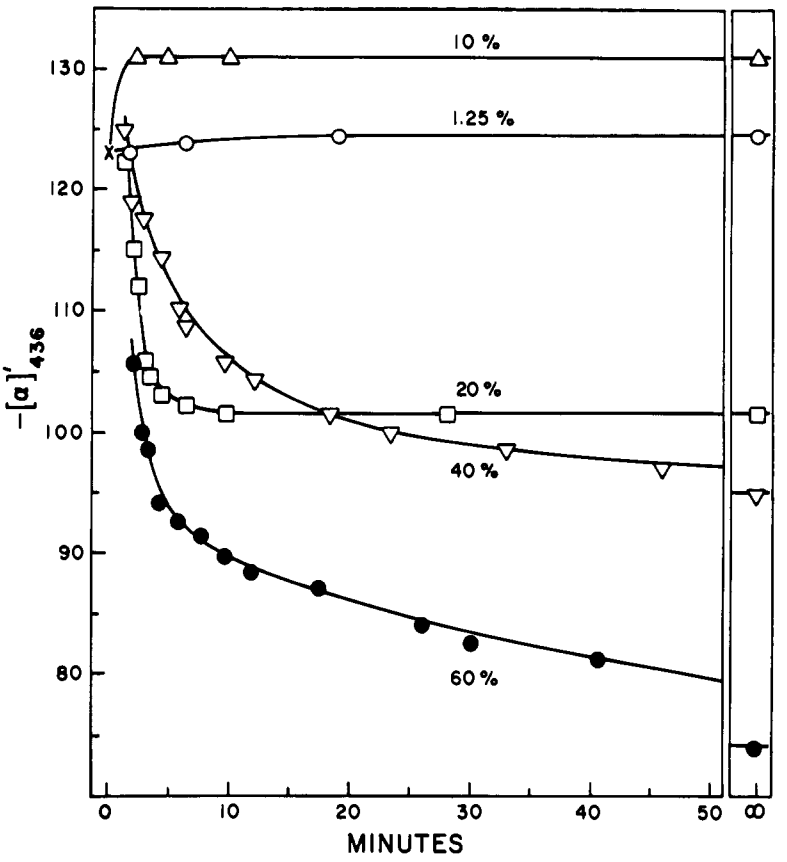

FIGURE 8: The rate of optical rotation change of DIPCT in chloroethanol at the indicated volume per cent concentrations. The symbol (x) represents the rotation of DIPCT in water.

chloroethanol, b_0 became more negative.

Reversibility of the Reactions. DIPCT in 30 or 77 vol. % chloroethanol was incubated for 72 hr at 30°. The samples were then dialyzed for 36 hr in the cold *vs.* 1 mM HCl. Comparison with the specific rotation of untreated DIPCT indicated that reversibility of the alcohol-treated protein was 95–99% complete.

Change in Viscosity. The change in a_0 and b_0 as the chloroethanol concentration was increased was attended by an increase in the reduced viscosity (Figure 6). The change was abrupt once the organic solvent concentration exceeded 10 vol. %.

Rate Studies. The ionic strength was an important variable in determining the rate and course of the spectral changes and the final equilibrium value of the molar difference extinction coefficient at 231 mμ. As an illustration of this effect, the time course of the spectral change at 293 and 231 mμ of DIPCT in 30 vol. % chloroethanol at an ionic strength of 0.08 is shown in Figure 7. At both spectral regions the maximum decrease in absorbance was reached in a very short time followed by a reversal of the change at 231 mμ to a more positive value *without a similar concomitant change in the region of specific tryptophan absorption.* The equilibrium value at 231 mμ, measured at 50 and 66 hr, was considerably more positive than that obtained at the same chloroethanol concentration but at the ionic strength of about 0.001. This effect of an increased ionic strength, *i.e.*, a reversal of the initially attained decrease in absorbance at 231 mμ to more positive values, has also been observed at other chloroethanol concentrations and a more thorough study of this phenomenon is being pursued (C. J. Martin and G. M. Bhatnagar, results to be published).

At low ionic strengths (*ca.* 0.001), a reversal of the difference absorbance at the low-wavelength region was not observed at a chloroethanol concentration of 40 vol. % or less (Figure 7). However, at 60 vol. %, the rate curve showed first a decrease in absorbance followed by a reversal to a more positive value (data not shown). The entire reaction was complete in about 150 sec and the final ΔA value was compatible with its equilibrium position as depicted in Figure 5. At still higher alcohol concentrations, changes in absorbance were too fast to measure.

At all chloroethanol concentrations (ionic strength, 0.001) spectral changes were complete in less than 10 min and final values for ΔA at all wavelengths remained unchanged within about a 20-hr period. Longer incubation times produced some additional slight change. This was traced to a change in the reference solution (DIPCT in water at pH 4.0) due to autol-

ysis (slight) despite the low concentration of active enzyme present (0.3–0.5%).

In the above rate studies, the difference absorbance from 400 to 310 mμ remained zero. This would appear to negate light-scattering effects as responsible for the reversal phenomenon of the difference absorbance in the 230-mμ region.

The time course of the optical rotation changes is shown in Figure 8. At low alcohol concentrations, the direction of change was to a slightly greater levorotation. At 20 vol. % chloroethanol and higher, the rotation became more positive with time. Because of technical limitations, observation of the reactions prior to about 2 min after zero time was impossible. However, inspection of Figure 8 shows that the curves obtained in 20 and 40 vol. % chloroethanol extrapolate to values higher than the zero time point which represents the specific rotation of DIPCT in aqueous solution. This is also indicated from first-order plots of the reactions, including that in 60 vol. % chloroethanol. From the intercept values of such plots and from the final equilibrium values for the specific rotation at 436 mμ, the curves obtained in 20, 40, and 60 vol. % chloroethanol apparently had an "initial" $[\alpha']_{436}$ at "zero" time of about −150°. This roughly corresponds to the value obtained in approximately 4.2 M urea or 2.3 M guanidinium chloride and which, by reference to Figures 1 and 3, produce only a partially denatured state. There is thus the strong inference that at about 20 vol. % chloroethanol and above, the change in optical rotation becames more levorotatory before the reaction to more positive values occurs.

The rate of spectral changes fitted apparent first-order kinetics. Likewise, the optical rotation change with time at 20 vol. % alcohol was also a first-order process. At higher concentrations of the organic solvent, the change in optical rotation was biphasic and could be analyzed as representing two simultaneous first-order reactions.

A summary of available rate constant data is given in Table I. All spectral rate constants (for the decrease in absorbance) are greater than the constants for the change in optical rotation to less levorotatory values. An increase in ionic strength serves to accelerate the rate of absorbance change.

In 40 and 60 vol. % chloroethanol, about 75% of the total change in optical rotation was associated with the fast reaction. Thus, the rate curves for DIPCT in 40 and 60 vol. % chloroethanol shown in Figure 8 are essentially those of the slow reaction and accounts, for example, for the crossover of the 20% line by the 40% line in the approach of the latter to its lower equilibrium value.

Temperature Transition Studies. In high concentrations of either urea or guanidinium chloride, the optical rotation of DIPCT decreased monotonically with an increase in temperature (Figure 9). This corresponds to the effect of temperature on the optical activity of a random chain and a similar result was reported by Schellman (1958b) for both CT and ribonuclease in 8 M urea. At the lower urea concentration of 3 M, wherein from the data of Figures 1 and 2 the protein is only slightly denatured, a temperature transition occurred with midpoint at about 45°. It will be noticed that this thermally induced transition does not result in a final optical rotation change equivalent to that obtained in high concentrations of either urea or guanidinium chloride. Rather, the change is of smaller magnitude and since it approximates that seen in the thermal transition of DIPCT at pH 2.0 in aqueous solution (Havsteen *et al.*, 1963; C. J. Martin and G. M. Bhatnagar, unpublished data) the process involves the conversion of state A (native state) to state B, a partially denatured state (Biltonen and Lumry, 1965). In water and in the absence of salt, the rotation remained constant to at least 45°.

Temperature transitions were also observed from measurements of the effect of temperature on the optical rotation of DIPCT in chloroethanol–water solutions (Figure 9). The presence of some ordered structure that could be "melted out" by heating was also indicated by the detection of spectral transitions at either 291 mμ or in the 230–235-mμ region. The results were closely equivalent to the polarimetric results and the change in $-\Delta\epsilon_M$ at 291 mμ was about 2600 through the transition region. The effect of temperature on both the spectral and optical rotation changes was not completely reversible. Upon cooling, only 80–95% reversibility was attained.

Experiments with Tryptophan and a DIPCT Digest. In all concentrations of chloroethanol, the difference

TABLE I: Rate Data for the Spectral and Optical Rotational Changes of DIP-Chymotrypsin in Chloroethanol–Water Solutions at pH 4.15 and 30°.

Observable	λ (mμ)	Chloroethanol (vol. %)	$k \times 10^4$ (sec^{-1})
Difference absorbance[a]	231	20	245
		30	685
		40	1725
		30[b]	*Ca.* 1900
	293	30[b]	*Ca.* 1950
Optical rotation[c]	436	20	96
		40	115[d] (70)[e]
			6.5[f] (30)[e]
		60	135[d] (78)[e]
			3.6[f] (22)[e]

[a] For the decrease in absorbance. [b] Ionic strength, 0.08. The ionic strength for all other rate measurements was about 0.001. [c] For the change to less levorotation. [d] For the fast reaction. [e] The per cent of total optical rotation change associated with the reaction. [f] For the slow reaction.

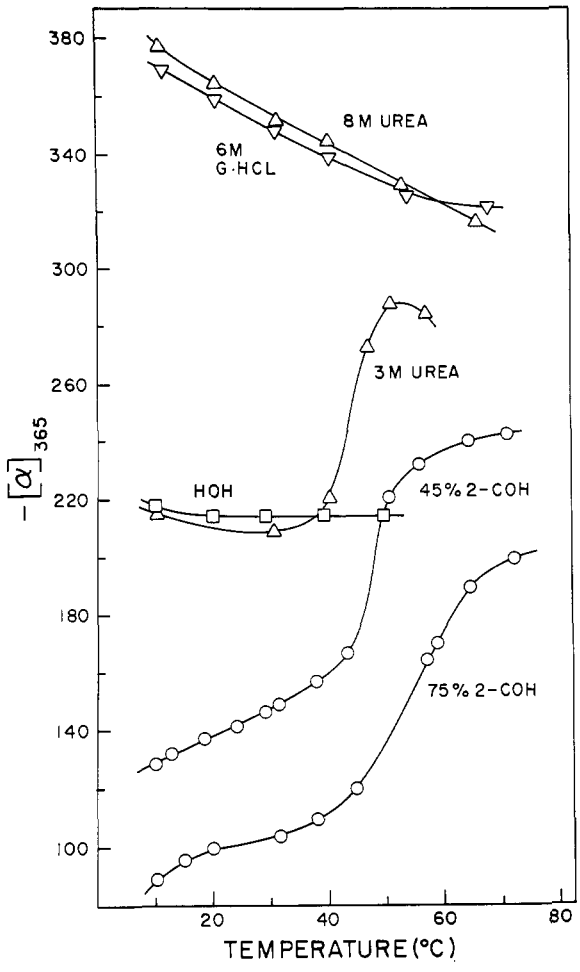

FIGURE 9: The effect of temperature on the optical rotation of DIPCT in the indicated solvents.

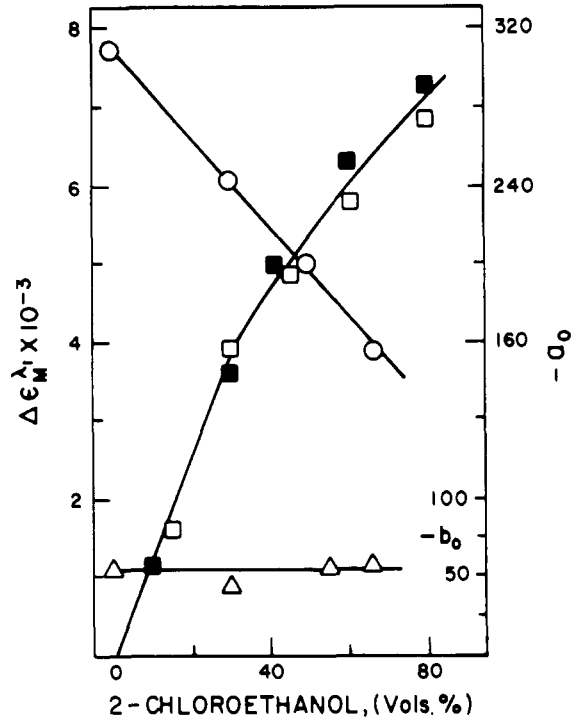

FIGURE 10: The effect of chloroethanol concentration on the Moffitt dispersion parameters and spectral changes of a DIPCT digest and the spectral changes of tryptophan. (○) change in a_0; (△) change in b_0; (□) change in apparent molar difference extinction coefficient for the DIPCT digest in the region of tryptophan absorption; (■) change in the molar difference extinction coefficient (times seven) of tryptophan.

spectrum of the DIPCT digest was that of a typical red-shift spectrum with major peaks of positive absorbance at 291–292, 283.5–284, and 229–230 mμ. In Figure 10, the increase in the *apparent* $\Delta\epsilon_M$ in the region of tryptophan absorption is plotted against the chloroethanol concentration. Data for the effect of the same solvent on the spectral properties of tryptophan (actual values multiplied by seven) at 290.5 mμ are also presented.

The variation in a_0 and b_0 for the DIPCT digest in varying concentrations of chloroethanol is also shown in Figure 10. The value of b_0 remained invariant and a_0 became more positive. These changes were *not* time dependent.

Experiments with Muramidase. Difference spectral measurements of muramidase in chloroethanol–water solutions *vs.* muramidase in water revealed major peaks at 300, 292.5, 285, 239–240, and 232 mμ. The variation in intensity of the negative maxima at 292.5 and 239–240 mμ with increasing chloroethanol concentration is given in Figure 11. For this protein, the changes at both peak positions were coincidental. The data obtained in the 290-mμ region agree reasonably well with that of Hamaguchi and Kurono (1963a). The change in $\Delta\epsilon_M$ with increasing chloroethanol concentration is characterized by a curve composed of segments with initially a positive, than a negative, and finally a positive slope. This contrasts with results obtained for DIPCT in the region of specific tryptophan absorption (see Figure 5).

From the data of Hamaguchi and Kurono (1963b), one can approximately determine that for the denaturation of muramidase in guanidinium chloride, the total blue shift (corrected for solvent effects) is attended with a value for $-\Delta\epsilon_M$ of 4700 at 292 mμ. This is approximately three times greater than the comparable blue-shift effect determined from the data of Hamaguchi and Kurono (1963a) for muramidase in chloroethanol–water solution. Using our data in Figure 11, the difference would be only 1.7-fold.

Muramidase contains six tryptophan residues per mole (Canfield, 1963) and, in the native state, about 4 equiv appears to be exposed to the solution environment (Hamaguchi and Kurono, 1963b; Herskovits, 1965). From the value given above for the total blue spectral shift that occurs upon denaturation of mur-

amidase in guanidinium chloride, the transfer of a tryptophan residue from its internal "buried" position to the external environment is associated with a value for $-\Delta\epsilon_M$ of 4700/2 or 2350. This is very close to the denaturation blue shift per tryptophan residue of both CT and DIPCT.

Discussion

In either urea or guanidinium chloride, CT and DIPCT undergo alterations in physical properties that are characteristic of what is commonly considered representative of protein denaturation reactions. The spectral changes are, as the denaturant concentration is increased, characterized by an initial red shift followed by blue-shift effects as contributions from the unfolding process predominate. If the denaturant concentration becomes sufficiently high, a second red spectral shift is seen due presumably to solvent effects on all exposed chromophores. Similar results have been observed in the denaturation of muramidase (Hamaguchi and Kurono, 1963b) and ribonuclease (Bigelow, 1964) by guanidinium chloride.

Denaturation was also attended by an increase in levorotation and by changes in the Moffitt dispersion parameters such that a_0 became more negative and b_0 more positive. Furthermore, heating of a solution of DIPCT in high concentrations of either urea or guanidinium chloride revealed a negative temperature coefficient corresponding to the effect of temperature on the optical rotation of a random coil (Schellman, 1958b).

Previous studies (Martin, 1964; Martin and Bhatnagar, 1966) of the kinetics of denaturation of either CT or DIPCT in urea or guanidinium chloride have demonstrated that changes in either difference absorbance or optical rotation were unidirectional. At all spectral peak positions, the difference absorbance became more negative, *i.e.*, the denatured state had less absorbance than the native state, and the optical rotation change was to a more levorotatory value. All kinetics were apparent first order and the spectral changes were synchronous with the changes in optical rotation. Equilibrium measurements of spectral changes as a function of either urea or guanidinium chloride concentration have also shown that the molar difference extinction coefficient at all peak positions occurs in phase and that the optical rotation changes are coincidental with these processes (Martin and Bhatnagar, 1966). Thus, the results are explicable with but a single cooperative unit participating in the denaturation reaction of CT and DIPCT.

In contrast to the behavior of proteins in urea and guanidinium chloride, where one is in general concerned with defining the parameters of the reaction from the native (folded) to the denatured (unfolded) state, the situation in chloroethanol–water solutions is more complex. The contrasting behavior of DIPCT in this organic solvent–water system to that in the usual denaturants can thus best be appreciated by a summation of the differences encountered.

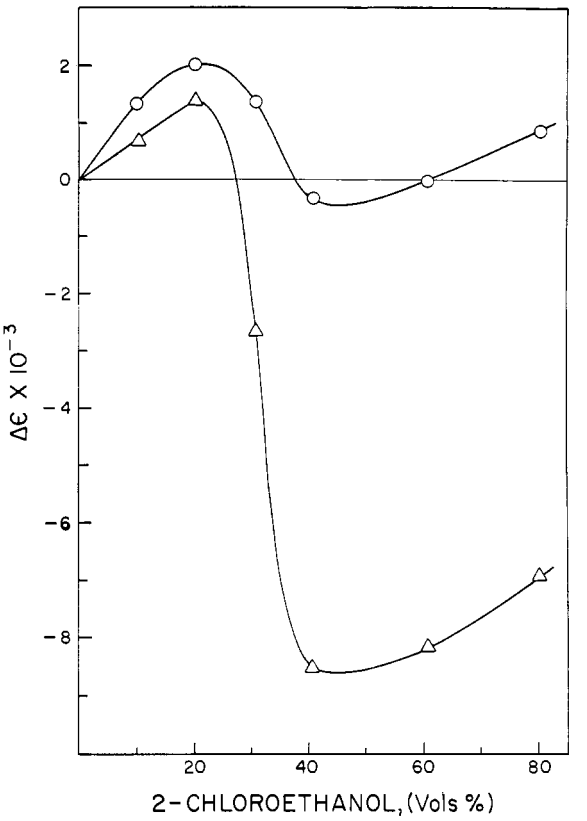

FIGURE 11: Variation in the molar difference extinction coefficient of muramidase with chloroethanol concentration. (○) change at 292.5 mμ; (△) change at 239–240 mμ.

Considering first the spectral changes, it was noted that they became anomalous as the concentration of chloroethanol was increased. In particular, the difference absorbance in the region of tryptophan absorption remained unchanged from about 20 to 70 vol. % chloroethanol. This change, *i.e.*, the absence of a red shift after the apparent maximal blue shift had occurred, was completely out of phase with that which occurred in the region of 230 mμ (see Figure 5). This behavior is at variance with the data from other experiments. For example, the spectral changes for muramidase at either high or low wavelengths were in phase and first positive, then negative, and finally more positive as the alcohol concentration was increased (see Figure 11). Furthermore, red-shift effects in the region of 290 mμ were observed over a wide range of chloroethanol concentration when either tryptophan or a DIPCT digest were used. Thus, tryptophan residues exposed to a chloroethanol solution environment will give rise to solvent-induced bathochromic shifts.

The changes in the ORD parameters with increasing chloroethanol concentration were also unusual in the sense that b_0 became first more positive and then, above 30 vol. %, more negative. The a_0 parameter became slightly more negative before it became more

positive throughout the remaining range of alcohol concentration employed. Thus, the changes in a_0 and b_0 at higher chloroethanol concentrations were in a direction opposite that usually associated with denaturation. Although the change of the a_0 parameter to more positive values may in part be the result of solvent effects (Tanford, 1962) (see also the results with the DIPCT digest, Figure 10), the changes were time dependent and proceeded essentially independently of and after the spectral changes had occurred. It is also pertinent to note that the increase in b_0 to a more negative value above 30 vol. % chloroethanol must reflect some structural characteristic of the molecule dictated by the environment since the b_0 value of the DIPCT digest was unaffected by the presence of chloroethanol.

As mentioned previously, differences exist between the spectral changes of DIPCT and muramidase in chloroethanol–water solutions. However, the change in a_0 and b_0 for both proteins is qualitatively similar with the exception that a minimum for the change in b_0 of muramidase (see Hamaguchi and Kurono, 1963a) is not observed as the alcohol concentration is increased.

Certain complexities also exist in the kinetics of the reactions undergone by DIPCT in chloroethanol–water solutions. At low ionic strength (*ca.* 0.001), all spectral changes (to somewhat above 40 vol. % chloroethanol) were unidirectional and to a less absorbing state. This is typical of a denaturation reaction. At 60 vol. % chloroethanol, a slight reversal of the initial, small absorbance change (negative direction) was observed at 231 mμ but not in the 290-mμ region. Evidence of reversal phenomena was also observed in a study of the kinetics of the optical rotational changes. At the higher ionic strength of 0.08, the events leading to a decrease in absorbance were accelerated about threefold relative to that which obtained at an ionic strength of about 0.001. Furthermore, the relatively rapid decrease in absorbance was followed by a slow reversal of the absorbance change at 231 mμ to more positive values without a corresponding change in the 290-mμ region.

In one additional respect, the behavior of DIPCT in chloroethanol–water solutions differs markedly from that in urea or guanidinium chloride. At relatively high concentrations of the alcohol, the protein can undergo a thermal transition as seen both spectrophotometrically and polarimetrically. The changes are not trivial and would appear to represent a cogent argument for the presence of an ordered structure in such solution environments at temperatures below the transition.

On the basis of the above plural approaches to the transconformation reactions of DIPCT in chloroethanol–water solutions, a tentative interpretation of the sequence of events that occur can be attempted. At relatively low concentrations of the alcohol (to about 30 vol. %) the protein undergoes a denaturation reaction. This is supported by the increase in reduced viscosity, the change in the b_0 dispersion parameter, and equilibrium and kinetic measurements of spectral changes. The denaturation process, however, is only partial. The spectral change in both the region of indole absorption and the 230-mμ region is much less than that which obtains in high concentrations of urea or guanidinium chloride. In the latter two denaturants, $-\Delta\epsilon_M$ for the total blue shift corrected for solvent effects is 10,000 at 293 mμ and 35,000 at 231 mμ. Apparent comparable corrections of similar data obtained in chloroethanol–water solutions would yield 10,000 (in the 290-mμ region) and 26,500 (in the 230-mμ region) for $-\Delta\epsilon_M$. The exact agreement of the one and the approximate agreement of the other value to that obtained in urea and guanidinium chloride is considered fortuitous and not to be interpreted as indicative of a close approach to the fully unfolded state. This viewpoint is supported by the observation of a temperature transition as detected by the change in tryptophan absorption, the constancy of tryptophan absorption between 20 and 70 vol. % chloroethanol, and the considerable discrepancy between the total blue shift of muramidase in chloroethanol–water relative to guanidinium chloride (see Results).

Above a chloroethanol concentration of about 30 vol. %, one can infer that some process involving structural ordering occurs. This concept has support in the optical rotatory dispersion behavior, spectral changes, and the demonstration of temperature transitions. Although it could be inferred from equilibrium measurements, kinetic results have provided reasonable evidence for the conclusion that the path from the native to the ordered state in high chloroethanol concentrations must involve a partially denatured state as an intermediate. It is not meant to imply, however, that the final ordered state is attained by a reversal of the denaturation reaction or that it represents a hyperfolded structure relative to the native state.

In contrast to the synchrony of all rate processes for the unfolding reactions of DIPCT (and CT) in urea and guanidinium chloride, the events in chloroethanol–water solution occur almost sequentially. For example, in 40 vol. % chloroethanol, the spectral change leading to a decrease in absorbance is 99% complete in about 28 sec (7 half-times). The change in optical rotation leading to a decrease in levorotation is biphasic and essentially complete in about 420 (fast reaction) and 7500 sec (slow reaction). Thus, the spectral change can be considered to be complete when only 0.3% of the slow and about 7% of the fast optical rotation change has occurred. This suggests that at least three cooperative units participate in the transition from the native to the equilibrium state. With the assumption that the change in optical rotation associated with the fast and slow reactions is proportional to the size of the cooperative units involved and that the entire molecule participates in the transition, about 25% of the molecule is associated with the slow reaction and 75% with the fast reaction. Evidence has also been obtained that under conditions wherein one can measure both the decrease in absorption and

the *increase* in levorotation in chloroethanol–water solution, *i.e.*, both changes characteristic of a denaturation reaction, the spectral change is faster than the optical rotation change (C. J. Martin and G. M. Bhatnagar, results to be published). Scott and Scheraga (1963) have reported that different regions of the ribonuclease molecule also unfold during heat denaturation with different rate-limiting steps.

One other anomoly of the behavior of DIPCT in chloroethanol–water solutions concerns the constancy of the tryptophan difference spectrum from about 20 to 70 vol. % alcohol. This is at complete variance with the spectral changes of tryptophan, a DIPCT digest, and muramidase in similar solutions and of DIPCT in either urea or guanidinium chloride. This possibly implies that once the partially denatured state of DIPCT has been attained at about 20 vol. % chloroethanol, the environmental domain of the tryptophan chromophores remains essentially unaltered, *i.e.*, at least as regards detection of the process, until the alcohol concentration exceeds about 70 vol. %. Mechanistically, this could be regarded as the resultant of a process in which all tryptophan residues (exposed and buried) become or remain buried in regions collectively less apolar than that which prevails in the native state.

The question remains then as to what type of structure might prevail for DIPCT in relatively high concentrations of chloroethanol. Certainly, the assumption that this protein might assume a more helical configuration than the native state solely on the basis of its more negative b_0 value would be premature. A number of reports have appeared in the literature which suggests considerable caution in the casual acceptance of the b_0 parameter as a measure of the helical content of polypeptides (Katzin and Gulyas, 1964; Ruttenberg *et al.*, 1965), synthetic polyamino acids (Hooker and Tanford, 1964; Hanlon and Klotz, 1965; Fasman *et al.*, 1965; Cassim and Taylor, 1965; Hanlon, 1966), and proteins (Kronman *et al.*, 1965, 1966). The general tenor of the criticisms is directed to emphasizing that any factor which influences the rotatory strength of the electronic transitions generating Cotton effects in the ultraviolet region will affect the b_0 value. The results of Ruttenberg *et al.* (1965) are particularly disconcerting. These authors showed that the cyclic decapeptide tyrocidine B (among others) has a large negative b_0 value even though these polypeptides *cannot* form helical structures.

Recently, Sarkar and Doty (1966) have demonstrated that, relative to the "random coil" structures, both poly-L-lysine and copoly-L-lysine–L-tyrosine in the β form gave more positive a_0 values and more negative b_0 values. The magnitude of the effects, however, was less than obtains for these polymers in the helical configuration. The occurrence of a β structure for DIPCT in high chloroethanol concentrations is thus also a possibility. Again, however, this would be by analogy only, and the same arguments voiced against the unreserved adoption of a helical structure would also apply.

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Shell Model Calculations of Rotational Diffusion Coefficients*

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ABSTRACT: Previous work has shown that it is possible to calculate the translational frictional coefficient of a complex structure by modelling the structure by a surface shell of spherical frictional elements. The shell model is here employed to calculate the rotational diffusion coefficients ($D^{\theta\theta}$) of structures with cylindrical symmetry, using theoretical formulations of Kirkwood and Hearst. Kirchoff's law for a sphere is obtained exactly, while $D^{\theta\theta}$'s calculated by this method are high by 8% or less compared to the exact Perrin results for prolate ellipsoids of revolution. Fair agreement with experiment on tobacco mosaic virus is obtained without explicitly introducing end effects in cylinders, which are shown to be small. The extension of tail fibers is shown to be necessary to obtain the $D^{\theta\theta}$ observed for the fast form of T2 bacteriophage by Maestre, if electron microscopic dimensions for the virus are used; but uniform expansion of the head by nearly a factor of two, or lengthening by a factor of three, is necessary to explain that observed for the slow form. Methods for extrapolation to a continuous surface distribution are discussed. It has been found empirically that coarse modelling of the surface, followed by shrinkage of the radii of the frictional elements by 50%, gives in all cases investigated a value of $D^{\theta\theta}$ which is within 6% of the shell model value.

Rotational diffusion coefficients, measured, for example, by flow or electrical birefringence or dichroism, or by fluorescence depolarization, provide an important source of information on the size and shape of macromolecules. In order for these coefficients to be interpreted, their dependence on size and shape for a model closely resembling the structure actually under study must be understood. The hydrodynamic models which have been studied theoretically up to now are ellipsoids of revolution (Gans, 1928; Perrin, 1934), rigid rods (Burgers, 1938; Broersma, 1960), random coils (Zimm, 1956), and wormlike chains of intermediate flexibility (Hearst, 1963). However, this range of structures by no means exhausts those encountered experimentally. In particular, many viruses have structures of considerable complexity, and it has seemed worthwhile to attempt to extend the methods of calculation of hydrodynamic properties to be able to deal with these more complex possibilities.

The basic theoretical foundation from which this extension takes place is Kirkwood's (1949, 1954; Riseman and Kirkwood, 1956) theory of irreversible processes in solutions of macromolecules. This theory treats macromolecules as being composed of identical frictional elements. Hearst (1963) has used the Kirkwood theory to obtain explicit expressions for the components of the rotational diffusion coefficient tensor (D) for a distribution of elements having cylindrical symmetry.

Previous work (Bloomfield *et al.*, 1967) has demonstrated that the Kirkwood theory can be combined with a particular method of modelling by small frictional elements to calculate with good accuracy the translational frictional coefficients of large structures of quite arbitrary shape. This method of modelling, called the "shell model," represents a particle of given shape by an assembly of small spherical frictional elements covering a surface of that shape. The hydrodynamic properties of the assembly of frictional elements will be close to those

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