Unfolding Reactions of Proteins. I. Kinetic and Equilibrium Measurements of Diisopropylphosphorylchymotrypsin and Chymotrypsinogen in Urea*

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ABSTRACT: The effect of urea on diisopropylphosphorylchymotrypsin and α-chymotrypsinogen has been investigated with respect to both the extent and rate of denaturation. Both proteins are denatured at essentially the same rate. Kinetically and thermodynamically, diisopropylphosphorylchymotrypsin is more stable than chymotrypsin.

A possible mechanism for the greater stability of diisopropylphosphorylchymotrypsin has been proposed which involves hydrophobic bonding and, as a direct consequence of this type of interaction, an increase in hydrogen bonding. The presence of the diisopropylphosphoryl moiety appears to stabilize the entire unit that unfolds in urea. Changes in difference absorbance at either 293, 285.5, or 231 mμ developed in phase with an increase in urea concentration. From kinetic measurements in 8 M urea, all rate data fitted apparent first-order kinetics with spectral changes at 293 and 231 mμ and optical rotational changes coincidental over the pH range 4–9. At either pH 4 or 7, the denatured state of diisopropylphosphorylchymotrypsin is characterized by a large blue spectral shift with $-\Delta\epsilon_M$ (corrected for solvent effects) at 293, 285.5, and 231 mμ, 10,000, 6750, and 35,000, respectively. On the basis of blue shift effects for the protein and spectral perturbation data of model compounds in solvents of varying refractive indices, it has been suggested that the difference absorbance peak at 231 mμ may contain substantial contributions from an environmental effect on the aromatic chromophores.

In a previous paper (Martin, 1964) the effect of high concentrations of urea and guanidinium chloride on the rate and extent of inactivation, spectral changes, and optical rotation of α-chymotrypsin was examined as a function of pH. All measurable reactions fitted apparent first-order kinetics. In urea, the rates of difference absorbance changes were synchronous at all peak positions and equal to the rate of inactivation (reversible plus irreversible) and the rate of optical rotation change. It was, therefore, concluded that the denaturation of the protein did not involve the sequential disruption of tertiary and secondary structural elements of the molecule and furthermore, that different regions of the molecule did not denature at different rates. In other words, the unfolding reaction apparently involved a single cooperative unit.

In guanidinium chloride, denaturation occurred much faster than in urea and the inactivation that attended the denaturation process, studied between pH 5 and 9.5, was completely reversible upon dilution into 1 mM HCl. This contrasted markedly with the behavior of the enzyme in urea wherein complete reversibility of inactivation could only be achieved upon dilution if the pH of denaturation was 5 or lower. It was determined that the irreversible changes observed were undoubtedly due to autolysis of the enzyme. In a general sense, the extent of such reactions was inversely related in a complex manner to the rate of denaturation.

Calcium ions considerably reduced the rate of chymotrypsin denaturation in urea but, at any pH above 5, increased the extent of irreversible reactions. Below a pH of about 4.5, the protective effect of this cation on the denaturation reaction disappeared. Stabilization toward denaturation was also effected by the addition of indole or 3-indolepropionate.

During the course of these studies, it was observed that diisopropylphosphoryl- (DIP) chymotrypsin[1] was more stable toward urea denaturation than was chymotrypsin. This paper is thus concerned with an investigation of the rate and extent of denaturation of DIP-chymotrypsin in urea. The denaturation of α-chymotrypsinogen and diphenylcarbamyl-(DPC) chymotrypsin has also been studied.

Experimental Procedure

The α-chymotrypsin preparations (Lot No. CDI-

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[1] Abbreviations used: DIP, diisopropylphosphoryl; DPC, diphenylcarbamyl; CTG, chymotrypsinogen; RNAase, ribonuclease.

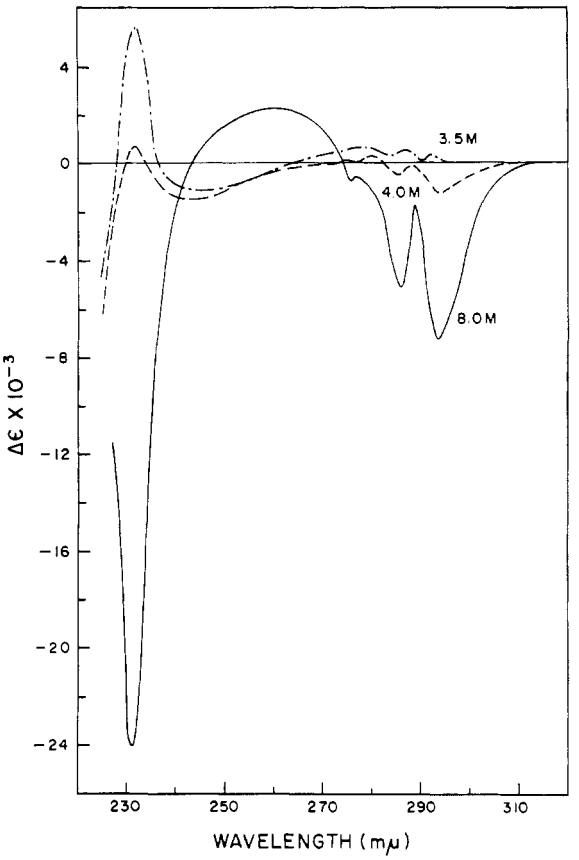

FIGURE 1: Difference absorption spectra of DIP-chymotrypsin at indicated concentrations of urea and containing 0.20 M $CaCl_2$ at pH 4.0 *vs.* the protein in 0.20 M $CaCl_2$ at the same pH.

6078, CDI-6093, and CDI-6096-7; three times crystallized, salt-free) and the α-chymotrypsinogen preparation (Lot No. CGC-541; five times crystallized, salt-free) were obtained from Worthington Biochemical Corp.

DIP-chymotrypsin was prepared by reacting diisopropylphosphofluoridate (Boots Pure Drug Co., England; distributed through Aldrich Chemical Co., Inc.) at 2.3×10^{-2} M with chymotrypsin (1.6×10^{-3} M) at pH 7.0 for 1 hr in the absence of added salt or buffer. The pH was then readjusted to 3.0 and the solution dialyzed exhaustively against 1 mM HCl at about 5°. A small amount of insoluble material was removed by centrifugation and the material either stored in the refrigerator (if to be used within a few days) or lyophilized. Null point potentiometric assay (DIP-chymotrypsin concentration, 0.5 mg/ml) at pH 8.0 and 30° with *N*-acetyl-L-tyrosine ethyl ester (0.01 M) as substrate in the presence of 0.10 M $CaCl_2$ showed a residual chymotrypsin activity of 0.3–0.5% for various preparations. Spectral measurements at both 293 and 231 mμ and optical rotational changes at 365 mμ as a function of temperature at pH 2.0 (ionic strength, 0.01) revealed only a single transition with all changes coincidental (C. J. Martin, N. B. Oza, and G. M. Bhatnagar, results to be published). Gross contamination of the DIP-chymotrypsin preparations by other material so as to yield a double

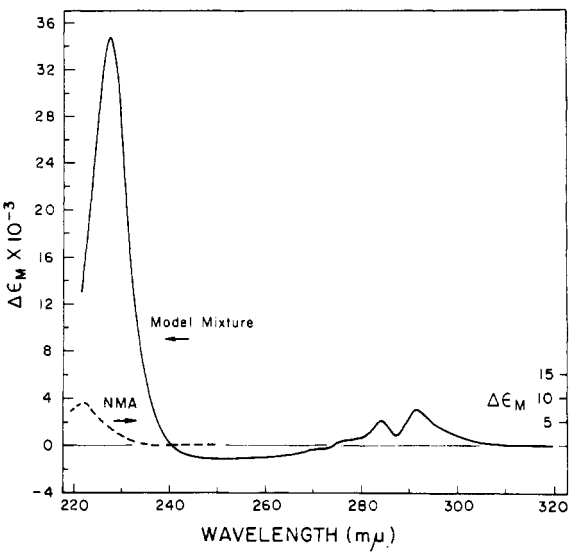

FIGURE 2: Difference absorption spectrum (solid line and left ordinate) of chymotrypsin model mixture at pH 4.0 (acetylated ethyl esters of tryptophan, tyrosine, and phenylalanine in mole ratios of 7:4:7) in 8 M urea *vs.* the same components in water. Dashed line (right ordinate), difference spectrum of *N*-methylacetamide in 8 M urea *vs.* compound in water.

transition as reported by Biltonen (1965) would therefore appear to be absent.

The preparation of DPC-chymotrypsin was modified from that of Erlanger and Cohen (1963). Chymotrypsin (4×10^{-4} M) was treated with diphenylcarbamyl chloride (8×10^{-4} M, Eastman Kodak, recrystallized from ethanol before use) at 25° for 90 min at pH 7.0 in the presence of 0.02 M $CaCl_2$, 0.02 M Tris buffer, and 20 vol % methanol. The solution was then adjusted to pH 3.0 and dialyzed against 1 mM HCl and lyophilized. Assay (DPC-chymotrypsin concentration, 1.3 mg/ml) *vs.* *N*-acetyl-L-tyrosine ethyl ester as above showed approximately 0.1% chymotrypsin activity.[2]

Urea was a Fisher Reagent product and was purified as previously described (Martin and Frazier, 1963a). Acetyl-D-tryptophanamide was obtained from the Cyclo Chemical Corp., acetyl-L-tryptophan ethyl ester and acetyl-L-phenylalanine ethyl ester from Mann Research Laboratories, and *N*-acetyl-L-tyrosine ethyl ester was a product synthesized in this laboratory. Deionized water was used throughout.

Difference spectra were obtained using a Cary Model 14 spectrophotometer fitted with thermostated reference and sample compartments. The reference solution (in reference beam) contained protein or model compounds at the same concentration, pH, and otherwise the same solution environment as the sample (in sample

[2] This value was obtained from the very initial slope of the rate curve. As the reaction continued, the rate increased and then decreased as substrate depletion was approached. Two separate preparations of DPC-chymotrypsin behaved similarly. It thus appears that under the conditions of assay used, a small amount of reactivation occurs.

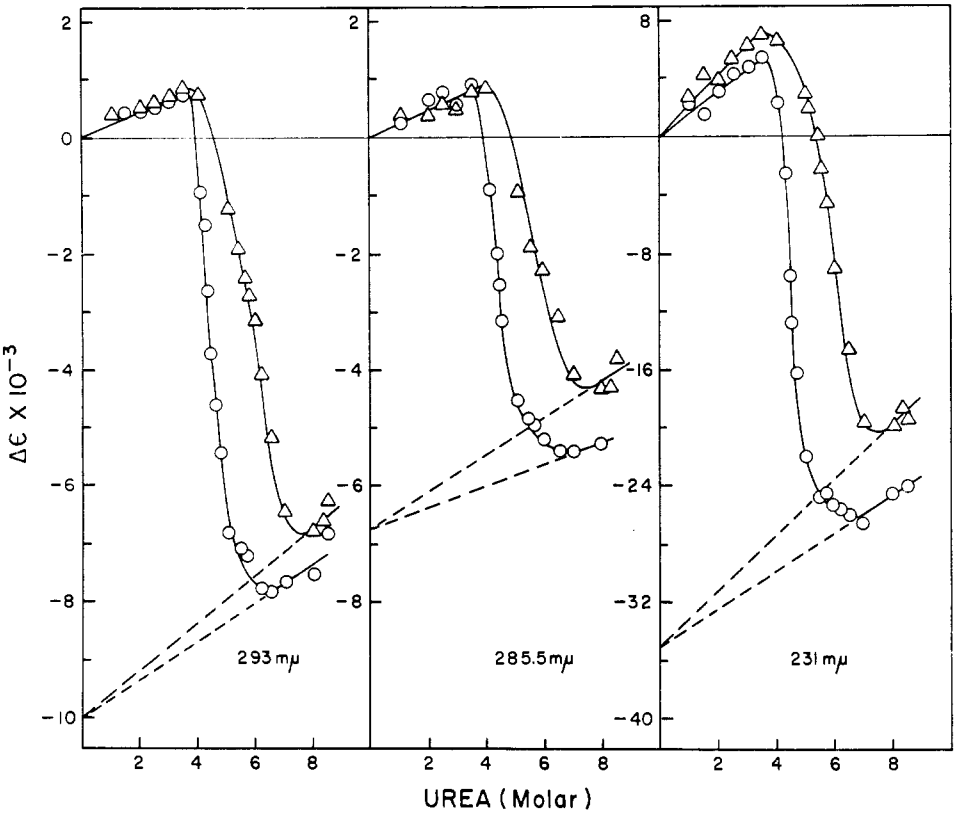

FIGURE 3: Variation in molar difference extinction coefficients at 293, 285.5, and 231 mμ with urea concentration of DIP-chymotrypsin in 0.20 M $CaCl_2$ at pH 4.0 (O) and 7.0 (Δ). Data given as average of four experiments at pH 4 and five experiments at pH 7

beam) but minus urea. One-centimeter light-path cuvets were used and Beer's law was obeyed within concentration ranges used. Correction for solvent absorption was obtained from the absorption spectra of appropriate urea solutions and the same pair of cuvets was used for the measurement of all spectra within any given experiment. Corrections were also applied for inequalities of the zero absorbance base line. In general, the gain and slit control settings of the spectrophotometer were adjusted so as to give a slit width no greater than 0.15 mm in the 290-mμ region and about 0.3–0.4 mm at the peak position in the low wavelength region near 230 mμ. Scan speed was 5 A/sec from 330 to 250 mμ and 2.5 A/sec from 250 to 225 mμ. Chart speed was 2 in./min. Protein concentration for measurements in the region of 230 mμ was less than 0.4 mg/ml. Molar difference extinction coefficients ($\Delta\epsilon_M$) were calculated using 25,000 as the molecular weight of all proteins.

Rates of denaturation were measured by following the change in difference absorbance at either 293 or 231 mμ or in optical rotation at either 436 or 365 mμ (Zeiss 0.005° spectropolarimeter). Details pertaining to these procedures were as previously described (Martin, 1954).

All solutions were filtered through a Millipore filter (pore size, 0.45 μ) prior to use to reduce scattering artifacts. Protein concentration was determined from the absorbance at 280 mμ and an extinction coefficient of 50,000 (Dixon and Neurath, 1957). Data for the dispersion of the refractive index of various solvents have been taken from Fasman (1963).

The pH of all solutions was measured with a Radiometer Model TTT-1 or 22 meter (G-202B glass and K4312 calomel electrodes) and, in the presence of urea, represents apparent, uncorrected values. All measurements were made at 30°.

Results

Difference Spectra. The equilibrium difference spectrum of DIP-chymotrypsin generated by solution of the protein in 8 M urea containing 0.20 M $CaCl_2$ at pH 4.0 showed prominent maxima of negative absorbance at 293, 285.5, and 231 mμ (Figure 1). A small spike at about 276 mμ and a broad envelope (positive absorbance) between 245 to 275 mμ were also present. The profile of this difference spectrum is thus similar to that shown by chymotrypsin (Martin, 1964) in the same solution environment. At neutral pH, the shape of the difference spectrum was similar but differences existed in the magnitude of the peaks (see below). At low concentrations of urea, the difference spectra were characterized by maxima at the same approximate wavelengths but of opposite sign. In contrast to the re-

sults at an urea concentration of 8 M, the generation of difference spectra up to an urea concentration of about 3.5 M was independent of time. This is consistent with a red shift spectrum and can be reasonably attributed (Yanari and Bovey, 1960; Bigelow and Geschwind, 1960) to a solvent perturbation effect on exposed chromophores due to the higher polarizability of the environment containing urea. As the urea concentration was further increased to 4.0 M a red to blue shift transition occurred (Figure 1). The maximum in the region of indole absorption (*ca.* 293 mμ) and phenol plus indole absorption (*ca.* 285 mμ) became negative but at the low-wavelength region, although the peak intensity at 231 mμ had decreased, the absorbance was still positive. The envelope of benzene absorption was essentially unchanged.

The contribution to the peak at 231 mμ is complex and is undoubtedly multiple in origin (Wetlaufer, 1962). As can be seen in Figure 2, a considerable contribution to this peak can arise from an environmental effect on aromatic amino acids. This spectrum was obtained by comparing a model mixture in 8 M urea composed of the acetylated ethyl esters of tryptophan, tyrosine, and phenylalanine in the molar ratios 7:4:7 of the amino acid residues contained in chymotrypsinogen (Wilcox *et al.*, 1957) *vs.* the same solutes in water. Perturbation of the spectrum of *N*-methylacetamide by 8 M urea resulted in a difference maximum at 222 mμ with $\Delta\epsilon_M$ 8.9.

The Effect of Urea Concentration. More detailed data of the effect of varying the urea concentration on the difference spectral changes of DIP-chymotrypsin at both pH 4 and 7 are shown in Figure 3. The magnitude of the solvent-induced red shift is defined by the initial slopes. In the region of 4 M urea, blue shift spectral effects begin to predominate as the protein unfolds and an inversion of the slope occurs. After the major portion of the transition has occurred, a second reversal of the curve occurs.

If one assumes that the spectral perturbation of all chromophores in the unfolded protein molecule is a linear function of the denaturant concentration, then extrapolation of the line defining the points above the transition zone at high urea concentration to zero urea concentration should yield $\Delta\epsilon_M$ values at the ordinate corrected for solvent effects. Bigelow (1964) has used this type of treatment to correct for solvent effects accompanying the denatured state of ribonuclease (RNAase) in guanidinium chloride. Since the only difference between sample and reference in the development of the difference spectrum is the presence of urea in the former, the corrected difference spectrum should be independent of pH (assuming the same equivalent state of denaturation with respect to the method of detection). Within experimental error, the results at both pH 4 and 7 yield the same ordinate value and appear to justify this treatment of the data.[3] Thus, solvent-corrected molar difference extinction coefficients for the denaturation of DIP-chymotrypsin in urea are −10,000 at 293 mμ, −6750 at 285.5 mμ, and −35,000 at 231 mμ.

The linearity of the data at low urea concentrations for the perturbation of the tryptophyl spectrum at 293 mμ (Figure 3) suffices as a good criterion that denaturation has as yet not occurred. Since the slope, within experimental error, is independent of pH one can assume that the tryptophyl equivalents exposed to the external environment are the same at both pH 4 and 7. This is in agreement with the results of Williams *et al.* (1965).

As the indole chromophore is essentially the sole contributor to the difference peak at 293 mμ, an estimate can be obtained from the data in Figure 3 and model compound studies of the tryptophyl equivalents exposed in the native protein. Perturbation of the spectrum of acetyl-D-tryptophanamide at pH 4.0 or 7.0 by varying concentrations of urea gave a slope at 291.5 mμ of 82.5 $\Delta\epsilon_M$ units-M^{-1}. From the data in Figure 3, the initial slope at 293 mμ is 225 $\Delta\epsilon_M$ units-M^{-1}. This yields an estimate of 2.7 tryptophyl equivalents exposed in the native protein at both pH levels. From the perturbation of the spectrum of acetyl-L-tryptophan ethyl ester by urea (slope, 69.4 $\Delta\epsilon_M$ units-M^{-1} at 291.5 mμ) the slightly higher value of 3.2 tryptophyl equivalents exposed is obtained. Inexplicably, data obtained from the perturbation of the model mixture (see Figure 2) indicated the exposure of 4.1 tryptophyl equivalents. The reason for this discrepancy is not apparent but certainly the value of about three equivalents is in agreement with the results of Williams *et al.* (1965) and Williams and Laskowski (1965) for chymotrypsinogen, chymotrypsin, and DIP-chymotrypsin. It is probable, however, from the results of both Oppenheimer *et al.* (1963) and of Williams *et al.* (1965) that a slightly greater burial of the tryptophyls may exist in DIP-chymotrypsin. Ignoring this possible difference, the value for $-\Delta\epsilon_M$ at 293 mμ of 10,000 then represents the value for the transfer of essentially four tryptophyl equivalents from the interior of the protein to the external environment. This amounts to 2500/residue and is in reasonable agreement with the results of Oppenheimer *et al.* (1963) wherein they obtained the value of 2800 for the same process as determined by comparison of the spectrum of chymotrypsin *vs.* digested chymotrypsin.

At 231 mμ, the initial slope at pH 7 is greater than at pH 4 and contrasts with the similarity in the initial slopes at both 293 and 285.5 mμ at either pH (Figure 3). This does not mean, however, that the peaks at the low and high wavelength regions develop out of phase. This can be seen by treatment of the data of Figure 3 in the following manner. Let $\Delta\epsilon_i$ define the initial slope of the data with the assumption that it extends linearly into the

[3] Admittedly, the lines defining the solvent-corrected values for $\Delta\epsilon_M$ represent subjective extrapolations. However, the reliability of the intercept values is strengthened by the fact that similar values have been obtained (no $CaCl_2$) for both the urea denaturation of chymotrypsin and for the denaturation of this enzyme and DIP-chymotrypsin by guanidinium chloride. In the latter denaturant and also for chymotrypsin in urea, the process of denaturation is complete at a much lower molar concentration and hence, the points defining the solvent effect extend over a wider concentration range with a consequent increase in the precision of intercept determination. These results will be presented in a subsequent paper (C. J. Martin and G. M. Bhatnagar).

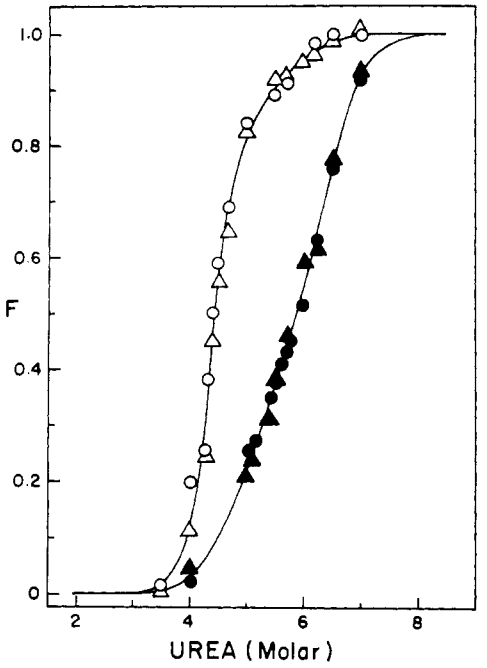

FIGURE 4: Variation in F, fraction of molecules (DIP-chymotrypsin) in the denatured state, *vs.* urea concentration (see text). Open symbols, pH 4.0; solid symbols, pH 7.0; circles, spectral change at 293 mμ; triangles, spectral change at 231 mμ.

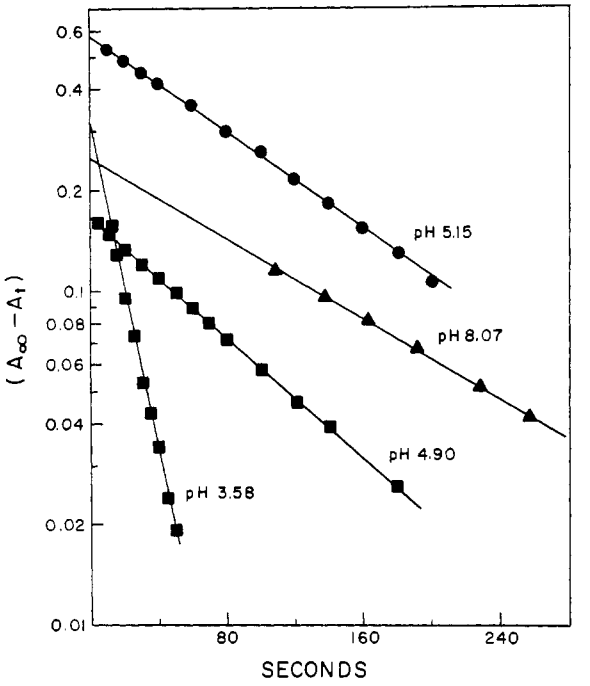

FIGURE 5: Adherence to apparent first-order kinetics of the denaturation of DIP-chymotrypsin in 8 M urea containing 0.20 M $CaCl_2$ at the indicated pH values. Data obtained by monitoring the change in difference absorbance at 293 mμ (■) or 231 mμ (●) or by the change in optical rotation at 365 mμ (▲).

region of high urea concentration. This would then define the magnitude of the red shift effect in the absence of denaturation. Let $\Delta\epsilon_f$ represent the final slope, as indicated by the lines extrapolated to zero urea concentration. Any point on these latter lines will represent the value of $\Delta\epsilon_M$ for the denatured protein at the particular wavelength but corrected for solvent effects only at the ordinate intercept. Then, if $\Delta\epsilon_e$ represents the experimental points containing contributions from both solvent and/or unfolding factors, the fraction, F, of the protein in the unfolded state at any urea concentration will be given by

$$F = (\Delta\epsilon_e - \Delta\epsilon_i)/(\Delta\epsilon_f - \Delta\epsilon_i)$$

Treatment of the data of Figure 3 at both 293 and 231 mμ by the above procedure resulted in a normalization of the data (Figure 4) and it can be seen that at the same pH, the change at 293 mμ develops in phase with that at 231 mμ. Although not shown, the changes at 285.5 mμ are also coincidental with the other spectral changes. At pH 4.0, DIP-chymotrypsin is half-unfolded (F, 0.5) at 4.4 M urea whereas the same state is attained at the higher urea concentration of 5.9 M at pH 7.0.

Although the plots in Figure 4 are not perfectly symmetrical, and intermediate states may be present, the results can provisionally be treated as a two-state cooperative transition of the native to the unfolded state. An apparent equilibrium constant, K, can be calculated for this process from the relation

$$K = F/(1 - F)$$

If one then assumes that the reaction

$$E_n + mU \rightleftharpoons E_dU_m$$

is descriptive of the reaction under consideration where m equals the moles of urea, U, combining with the native protein, E_n, to produce the unfolded state, E_dU_m, it follows that a straight line should be obtained by plotting $\log K$ *vs.* $-\log U$ (substituting molar concentrations for activities) with the slope equal to m. This was found to be approximately true over most of the transition range with m equal to 15 ± 1 at pH 4 and 9.5 ± 0.5 at pH 7. This high order with respect to urea is characteristic of other denaturation reactions. For example, at pH 7.35 and 25°, Barnard (1964a) has determined m to be 16 (equilibrium measurements) and 12 (kinetic measurements) for the urea denaturation of RNAase. Assuming that DIP-chymotrypsin would behave similarly to chymotrypsin, m would be about 6.5 from kinetic measurements near neutral pH (Martin and Frazier, 1963b). A pattern similar to the RNAase case is thus apparent.

Spectral Changes of Chymotrypsinogen and DPC-Chymotrypsin in Urea. Denaturation of chymotrypsino-

TABLE I: Molar Difference Extinction Coefficients for α-Chymotrypsinogen and Diphenylcarbamylchymotrypsin in 8 M Urea ±0.20 M $CaCl_2$.

Protein	pH	Ca^{2+}	$\Delta\epsilon_M$ 293 mμ	285.5 mμ	231–231.5 mμ
CTG[a]	4.0	−	7,600	5,300	23,150
		+	8,460	6,800	22,800
	7.0	−	7,720	5,520	22,800
		+	7,640	5,580	21,100
DPC-CT[b]	4.0	−	7,290	5,025	26,175
	7.0	−	7,500	5,370	28,700

[a] Average of three experiments. [b] Average of two experiments.

gen and DPC-chymotrypsin in 8 M urea gave essentially the same results as for the denaturation of DIP-chymotrypsin (Table I). The presence of calcium ions had no effect on the value of $\Delta\epsilon_M$ for the zymogen at any wavelength.

Kinetics of Denaturation. The rate of unfolding of DIP-chymotrypsin in urea as indicated by either the decrease in absorbance or the increase in levorotation fitted apparent first-order kinetics (Figure 5).

All rate constant data are plotted in Figure 6. The over-all curve profiles are similar to that found for chymotrypsin (Martin, 1964) with the exception that the values are considerably smaller. For example, the much slower denaturation rate for DIP-chymotrypsin in 8 M urea containing 0.20 M $CaCl_2$ can be compared with the rate constant *vs.* pH curve for chymotrypsin obtained under the same conditions and represented by the unbroken line in Figure 6.

Calcium ions depressed the rate of DIP-chymotrypsin denaturation above a pH of about 4.5. Below this pH, the rate constants obtained in the presence and absence of calcium ions were essentially the same.

The decrease in absorbance at 293 mμ was synchronous with the change detected at 231 mμ (Figure 6). The rate of optical rotational change also paralleled the spectral changes.

A few rate measurements were also carried out in the presence of indole (Figure 6). At the concentration level used, no effect on the rate of denaturation of DIP-chymotrypsin was observed.

In an attempt to gain some insight into the stabilization of chymotrypsin toward urea denaturation by the introduction of the diisopropylphosphoryl group, free energies of activation (ΔF^*) have been calculated as a function of pH from the rate constant data of Figure 6 using transition state theory. Comparable calculations have also been done using data for chymotrypsin as given in a previous paper (Martin, 1964). The free energies of activation for the denaturation of both proteins in 8 M urea in the presence and absence of 0.20

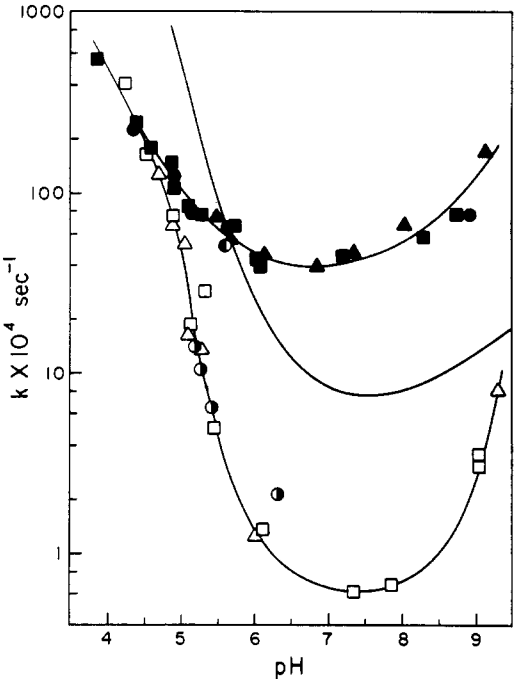

FIGURE 6: Apparent first-order constants for the denaturation of DIP-chymotrypsin in 8 M urea in the absence of (solid symbols) or in the presence of (open symbols) 0.20 M $CaCl_2$. Rate constants are calculated from the change in difference absorbance at 293 mμ (squares), 231 mμ (circles), and by the change in optical rotation at 365 mμ (triangles). Rate constants in the presence of 0.024 M indole were determined by the change in optical rotation in the presence of (◑) or absence of (◐) 0.20 M $CaCl_2$. The line without experimental points represents the effect of pH on the rate constant for the denaturation of chymotrypsin in 8 M urea plus 0.20 M $CaCl_2$ and has been taken from a previous paper (Martin, 1964).

M $CaCl_2$ over the pH range from 4.5 to 9.0 are tabulated in Table II. These values will contain contributions from the thermodynamic functions of the ionizable groups since the rate constants are markedly dependent on pH. In this connection, Marini and Behr (1964) have shown that the titration curve for DIP-chymotrypsin and chymotrypsin are identical within experimental error. Therefore, if one makes the not too unreasonable assumption that these and other contributions such as the degree of solvation of the activated complex are essentially the same for chymotrypsin as for DIP-chymotrypsin, then, by taking the difference between ΔF^* for DIP-chymotrypsin and ΔF^* for chymotrypsin in the absence of calcium chloride, an estimate can be obtained for the stabilization that the introduction of the DIP group contributes to the ΔF^* of the activated complex.

On somewhat more tenuous grounds, one can also obtain an estimate of the contribution to ΔF^* by the presumed binding of calcium to both proteins. Previous

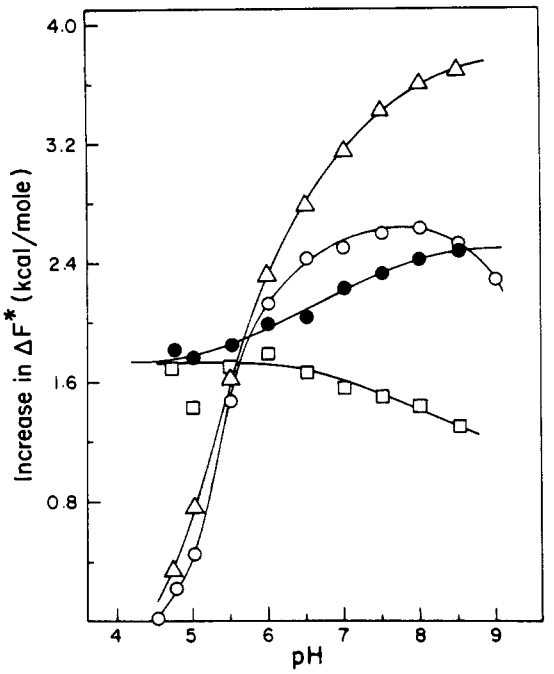

FIGURE 7: The increase in the free energy of activation (ΔF^*) for the denaturation of chymotrypsin in 8 M urea with pH as the operational result of the presence of 0.20 M $CaCl_2$ [Δ (b minus a, Table II)] and the introduction of the DIP group [(● (c minus a, Table II) or □ (d minus b, Table II)]. The symbol, ○, represents the increase in ΔF^* for the denaturation of DIP-chymotrypsin due to the presence of 0.20 M $CaCl_2$ (d minus c, Table II).

TABLE II: Free Energies of Activation for the Denaturation of Chymotrypsin and DIP-Chymotrypsin in 8 M Urea ±0.20 M $CaCl_2$ at 30.0°.

	ΔF^* (kcal/mole)[a]			
pH	CT (a)	CT + Ca (b)	DIP-CT (c)	DIP-CT + Ca (d)
4.50			20.21	20.21
4.75	18.60	18.94	20.40	20.62
5.0	18.85	19.61	20.59	21.03
5.5	19.07	20.68	20.91	22.38
6.0	19.14	21.46	21.12	23.24
6.5	19.10	21.89	21.21	23.54
7.0	18.99	22.14	21.21	23.69
7.5	18.82	22.24	21.15	23.73
8.0	18.60	22.20	21.01	23.63
8.5	18.37	22.06	20.84	23.35
9.0			20.54	22.82

[a] Calculated from the smooth curves of k *vs.* pH plots for chymotrypsin denaturation (Martin, 1964) and from the curves for DIP-chymotrypsin as given in Figure 6.

kinetic data for the denaturation of chymotrypsin in urea containing calcium ions were consistent with the possibility that the binding of calcium to the protein was on an equimolar basis (Martin and Frazier, 1963b). By analogy, this will be presumed to also obtain with DIP-chymotrypsin since certainly the presence of calcium ions does decrease considerably the rate of denaturation. The results of such substractive processes are presented graphically in Figure 7.

Rate of Denaturation of Chymotrypsinogen. Measurement of the rate of change of optical rotation of chymotrypsinogen in 8 M urea containing 0.20 M $CaCl_2$ gave apparent fit to first-order kinetics. Data were obtained over only a limited pH range but were sufficient to show that the rate constants were very similar to those for DIP-chymotrypsin under comparable conditions (Table III). As was found for DIP-chymotrypsin, indole had no effect on the rate of denaturation since all of the data in Table III can be fitted to the same smooth curve.

TABLE III: Apparent First-Order Rate Constants for the Denaturation of α-Chymotrypsinogen in 8 M Urea Containing 0.20 M $CaCl_2$ in the Absence and Presence of Indole as Determined by the Change in Optical Rotation at 436 mμ.

	$k \times 10^4$ sec^{-1}	
pH	No Indole	With Indole (0.024 M)
4.96	47.7	
5.07	30.3	32.1
5.13	26.7	25.1
5.40		14.2
6.15	2.85	

It has been previously shown that indole and 3-indolepropionate decreased the rate of urea denaturation of chymotrypsin (Martin, 1964). Since neither DIP-chymotrypsin nor chymotrypsinogen showed a decreased denaturation rate in the presence of indole, the conclusion seems reasonable that the increased stability of chymotrypsin in the presence of these compounds is due to their binding only at the catalytic site.

Discussion

It is by now a classical concept in enzyme chemistry that an enzyme can be protected by its substrate against denaturation. Many examples of such phenomena are to be found in the literature and a review by Grisolia (1964) summarizes much that has been done in this area including the more recently recognized and opposite phenomenon of the inactivation of an enzyme by its substrate or substrate analogs.

Introduction of a covalently bonded group or pseudo-substrate into a protein has also been shown to either enhance or decrease stability. For example, attachment of a carboxymethyl group to either the 12- or 119-histidine in RNAase results in a decrease in the rate of urea denaturation below a pH of about 5.5 (Yang and Hummel, 1964; Barnard, 1964a). Above a pH of 7.5, the 12-histidine derivative was the most rapidly denatured form with the 119-histidine derivative and RNAase being denatured at the same rate (Yang and Hummel, 1964). It was also shown that at pH 5, both derivatives have a lower transition temperature than RNAase. At pH 8, the transition temperature of the 12-histidine derivative was lower than that of either the 119 derivative or RNAase. Thus, a parallelism exists in this case between kinetic and thermodynamic stability.

Modification of chymotrypsin to yield either the acetyl enzyme or DIP-chymotrypsin results in an increase in the transition temperature (Havsteen and Hess, 1963; Havsteen *et al.*, 1963). Diethylphosphoryl-chymotrypsin is also more stable toward heat than the unmodified enzyme (Cohen and Erlanger, 1960).

In this paper it has been shown that both DIP-chymotrypsin and chymotrypsinogen (at least in the acid pH range) are essentially kinetically equivalent in their rate of denaturation by urea and both are considerably more stable than chymotrypsin. The greater stability of the zymogen relative to chymotrypsin may be the result of its single polypeptide chain affording additional restraints to unfolding. Chymotrypsinogen is also thermodynamically more stable since its transition temperature at pH 2.0 is 43° (Eisenberg and Schwert, 1951; Brandts and Lumry, 1963) whereas that of chymotrypsin is about 33° (Havsteen *et al.*, 1963).

In conformity with the effect of calcium ions on the urea denaturation of chymotrypsin (Martin, 1964), this cation also decreased the rate of denaturation of DIP-chymotrypsin. Since calcium ions also decrease the urea denaturation rate of chymotrypsinogen (Chervenka, 1960) this might argue that a common binding site for this cation exists on all three proteins. It has already been shown that the effect of calcium (and strontium) is rather specific and not an ionic strength effect and that for both chymotrypsinogen (Chervenka, 1960) and chymotrypsin (Martin and Frazier, 1963b) the kinetic evidence was consistent with calcium combining with protein in a 1:1 mole ratio. By analogy, this result may also obtain with DIP-chymotrypsin.

Calcium ions do not protect either chymotrypsin (Martin, 1964) or DIP-chymotrypsin against urea denaturation below a pH of about 4.5. Above this pH, the stabilization due to calcium is reflected in a decrease in the apparent first-order rate constants for denaturation and in an increase in ΔF^* for both proteins. When values for ΔF^* for denaturation in the absence of calcium are subtracted from ΔF^* values for denaturation in the presence of calcium, the difference quantities obtained for both chymotrypsin and DIP-chymotrypsin approximately parallel each other up to a pH of about 6. At higher pH values, the curve levels off for the latter protein more rapidly than for chymotrypsin (see Figure 7). In fact, the increase in ΔF^* for the stabilization of DIP-chymotrypsin by calcium undergoes an inversion in slope at pH 8. If the mechanism of calcium stabilization against conformational changes of the protein is its binding to two anionic sites this should, as the data shows, be reflected in a pH dependence for the increase in ΔF^*. However, it would be naive to assume that as the presumed binding of calcium approaches completion that other types of intramolecular interactions, either favorable or unfavorable for stability, would not also occur. Further discussion of this phenomenon must, therefore, await more direct and definitive evidence.

The curve describing the increase in ΔF^* of chymotrypsin toward urea denaturation (calcium ions absent) due to the presence of the DIP group is much less dependent on pH and within the limitations of the data appears to be sigmoid in form (see Figure 7, solid circles). The shape and position of this curve in conjunction with other ancillary information suggest the following provisional interpretation for the greater stability of DIP-chymotrypsin relative to chymotrypsin.

It would appear reasonable to assume that the isopropyl groups of the DIP moiety could participate in hydrophobic bonding with other apolar side chains (whose strength of interaction might thereby also be increased) in the ring sequence of amino acid residues between half-cystines 42 and 58 [as numbered for chymotrypsinogen, Hartley (1964)].[4] Therefore, about 1.7 kcal/mole might be contributed to the value of ΔF^* for the denaturation of DIP-chymotrypsin by hydrophobic bonding. In the absence of other factors, this quantity should be pH independent. The pH-dependent quantity of about 0.8 kcal/mole (the increase from pH 4.5 to 8.5) might then arise from an increase in hydrogen bonding since Klotz and Franzen (1962) have shown that the strength of such bonds in model systems increases with an increase in the apolarity of the environment. Thus, if a possible donor and acceptor group were within the domain of influence created by an increase in hydrophobic bonding, the potential for hydrogen bonding to occur would be increased. From the midpoint (6.6) of the pH-dependent transition it is tempting to consider the imidazole group of a histidine residue as the likely candidate for the hydrogen bond acceptor. In fact, the data of Figure 7 closely resemble the acid half of the theoretical curve for hydrogen bond formation between histidine and the phenolic group of tyrosine (Scheraga, 1961) if allowance is made for the perturbation of the pK' of the imidazole group by a more apolar environment and hydrogen bonding (opposing effects). It has also been reported that the two abnormally ionizing tyrosines (Havsteen and Hess, 1962; Marini and Wunsch, 1963) and both histidines in DIP-chymotrypsin are less reactive to cyanuric

[4] Examination of a space-filling model of this portion of the amino acid sequence of chymotrypsin, with positions 40 and 57 occupied by histidine residues, reveals a cavity rich in hydrophobic side chains into which the DIP group, presumably attached to serine residue 195, could be accommodated.

fluoride and diazonium-1*H*-tetrazole, respectively, than their counterparts in chymotrypsin (Hachimori *et al.*, 1965). For both proteins, one histidine was less reactive than the other.

On the basis of the above discussion, the working hypothesis can be entertained that DIP-chymotrypsin is more stable than chymotrypsin because of an increase in hydrophobic bonding and, as a consequence of this type of interaction, by the added stabilization of hydrogen bonding. The possibility remains open, however, that the differences between the two proteins may have their origins in part or in whole to other and more complex interactions.

The kinetic evidence indicating a greater stability of DIP-chymotrypsin toward urea denaturation than chymotrypsin is corroborated in the thermodynamic sense by equilibrium measurements of spectral changes as a function of urea concentration. At pH 4, where calcium ions have no effect on the stability of either protein, chymotrypsin unfolds at a lower urea concentration than does DIP-chymotrypsin (C. J. Martin and G. M. Bhatnagar, results to be published).

When ΔF^* values for the denaturation of chymotrypsin in urea plus calcium chloride are subtracted from comparable data for DIP-chymotrypsin a curve different from that obtained in the absence of salt results (see Figure 7, squares). Up to a pH of about 6, the increase in ΔF^* is about 1.7 kcal/mole but as the pH is increased, the value decreases. This is in part the result of the difference contributed to the stability of the two proteins by the presence of calcium ions. The remainder may be the result of the increase in ionic strength (0.6) with attendent alterations in the interactions induced by the presence of the DIP group as a consequence of alteration in water structure, and hence hydrophobic bonding, and in electrostatic factors.

In the urea denaturation of DIP-chymotrypsin, the same rate constant, at any pH, describes both the spectral (at either 293 or 231 mμ) and optical rotational changes. The spectral change at 285.5 mμ, by analogy with results for chymotrypsin (Martin, 1964), is probably also synchronous with these changes. These facts would make it appear reasonable to conclude that the stabilization afforded to chymotrypsin by the presence of the DIP group is not a localized effect but rather extends to the entire unit participating in the unfolding process. In other words, there is no evidence that different portions of the molecule unfold with different rate-limiting steps such as has been reported by Scott and Scheraga (1963) for the heat denaturation of RNAase. Therefore, since biphasic rate curves have not been observed in the urea denaturation of DIP-chymotrypsin and spectral changes at two (and probably three) peak positions are synchronous with optical rotational changes, it would suggest that a cooperative unfolding of the molecule occurs. That the DIP group stabilizes the entire cooperative unit would appear to require that the unit size be approximately the same as for chymotrypsin. An indication that this is so has been obtained by Biltonen (1965) and Biltonen and Lumry (1965b) from measurements of thermally induced transitions. For chymotrypsinogen, the size of the cooperative unit is smaller (Brandts, 1964).

From equilibrium measurements, the unfolding of DIP-chymotrypsin in increasing concentrations of urea as detected by absorbance changes at 293 mμ is in phase with the changes at 285.5 and 231 mμ. From this one can conclude that although the spectral change at 231 mμ may be dominated by the same environmental effects (the possibility of hydrogen bonding is not excluded) on the aromatic chromophores that give rise to the spectral changes at the higher wavelength region (see below), all other factors contributing to the change at 231 mμ such as a conformation-dependent alteration in peptide bond absorptivity or absorbance changes due to histidine, cystine, etc. (Wetlaufer, 1962), also occur in phase with the other spectral changes. The coincidental nature of the spectral changes would therefore support the possible conclusion that the unfolding reaction was a two-state cooperative reaction. However, the lack of symmetry of the curves in Figure 4, particularly at the lower pH, would suggest that intermediate states at concentrations below about 8 M urea might be present. The existence of intermediate states for the denaturation of proteins in urea has also been suggested by Tanford (1964), Edelhoch and Lippoldt (1964), and Barnard (1964b).

As has been shown, DIP-chymotrypsin is both kinetically and thermodynamically more stable at pH 7 than at 4. However, since the kinetic data indicate no effect of calcium at a pH below about 4.5, the difference observed between the two pH values reflects, in addition to other factors, the absence of added calcium stabilization at pH 4. This is supported by the fact that a $\Delta\epsilon_M$ *vs.* urea concentration (no Ca^{2+}) plot for DIP-chymotrypsin at pH 4 is essentially the same as in the presence of calcium ions (C. J. Martin and G. M. Bhatnagar, results to be published).

The denaturation of DIP-chymotrypsin can progress *via* several transitions to the maximally unfolded state. For the case of chymotrypsin, the states involved have been designated by Biltonen and Lumry (1965a) as state A for the native protein; state B for the form produced by the low-temperature transition; state C for the form in high denaturant concentration; and state D for the form as in state C but with all disulfide bonds ruptured. The spectral data of this paper thus serve to define the values of $\Delta\epsilon_M$ at all peak positions for state C of DIP-chymotrypsin. The values reported also apply to chymotrypsin in urea and to DIP-chymotrypsin and chymotrypsin in guanidinium chloride (C. J. Martin and G. M. Bhatnagar, results to be published). From the spectral data of Williams *et al.* (1965), it would also appear that the $\Delta\epsilon_M$ values for state C, at least at 293 mμ, will also be equivalent to those of state D. Although data for chymotrypsinogen and DPC-chymotrypsin have only been obtained at a single urea concentration (8 M), it would appear likely that the denatured forms of these proteins probably also have the spectral characteristics of DIP-chymotrypsin in state C.

In the transition of DIP-chymotrypsin to state C at either pH 4 or 7, $\Delta\epsilon_M$ at 293 and 231 mμ (corrected for

solvent effects) was −10,000 and −35,000, respectively. The value for $\Delta\epsilon_M$ at 293 mμ represents a molar difference extinction coefficient for the transfer of probably four (see Results) tryptophyl equivalents from the interior of the protein to the aqueous environment. If it is assumed that the value of 2500/residue equivalent arises solely from a change in the environment from one of high to low polarizability, certain inferences can be drawn about the effective refractive index of the buried tryptophyl environments and, as a consequence, an approximation to the contribution of the peak magnitude at 231 mμ due to the same process.

From data obtained in this laboratory, it has been determined (from slopes of $\Delta\epsilon_M$ *vs.* solvent composition plots) that the transfer of either acetyl-L-tryptophan ethyl ester, acetyl-D-tryptophanamide, or a model mixture of chymotrypsin from 8 M urea (n^{20}_{289} 1.4433) to water results in an average $\Delta\epsilon_M$ at 291.5 mμ of −550. For the transfer of acetyl-D-tryptophanamide, tryptophan, or a DIP-chymotrypsin digest from 2-chloroethanol (n^{20}_{289} 1.4823) to water, $\Delta\epsilon_M$ per mole of tryptophan is −1880 (average value). If these $\Delta\epsilon_M$ values are plotted *vs.* the refractive indices at 289 mμ (the use of n values at 289 mμ rather than at 293 mμ will not affect the argument), it is found, assuming linearity of the relationship, that the experimental value of −2500 for the transfer of one tryptophyl equivalent from the interior of DIP-chymotrypsin to the aqueous solution fits the line at a refractive index of 1.50. Yanari and Bovey (1960) have stated that the observed spectral changes of proteins in the high-wavelength region can be accounted for if the effective polarizability of the hydrophobic regions describing the environment of the aromatic chromophores was equivalent to that of carbon tetrachloride. Extrapolation of the values for the dispersion of the refractive index of this solvent to 289 mμ (lowest wavelength listed was at 313.1 mμ) gives a value for n^{20} of 1.507. This is thus in essential agreement with the value as found above. The value of 1.50 will thus be used as the effective refractive index of the tryptophyl environments at 289 mμ.

The transfer of L-tryptophan from 2-chloroethanol (n^{20}_{265} 1.4940) to water yields $\Delta\epsilon_M$ equal to −6900 at 231 mμ. For the transfer of acetyl-L-tryptophan ethyl ester from 8 M urea (n^{20}_{265} 1.4572) to water, $\Delta\epsilon_M$ equals −3140. Plotting of these data against the refractive indices at 265 mμ (data were not available at 231 mμ) yielded a line whose other coordinate at n^{20} equal to 1.50 was −7500. This value, of course, is only an approximation and would be less than this if refractive indices at 231 mμ had been used for plotting purposes. However, the decrease due to this effect would be opposed by the dispersion (to 231 mμ) of the effective refractive index of 1.50 at 289 mμ. Sufficient data are not available, however, to attempt a sorting out of the effect of these parameters. Thus, within the limitations of the approximations involved, the transfer of four tryptophyl equivalents from the interior of DIP-chymotrypsin to an aqueous environment would give a value for $\Delta\epsilon_M$ at 231 mμ of 4(−7500) or −30,000. Admittedly, this value may be too high but the point to be made is that it can be a significantly large fraction of the experimentally observed value of −35,000 at 231 mμ.

Perturbation of the spectrum of *N*-methylacetamide by 8 M urea resulted in a red shift with a maximum at 222 mμ and with $\Delta\epsilon_M$ equal to 8.9. Since the absorption band of the peptide bond can be shifted about 10 mμ upon incorporation into a polymer state and due primarily to the presence of bulky side chains (Glazer and Rosenheck, 1962), a shift of only 9 mμ would place the solvent-induced difference peak of *N*-methylacetamide at 231 mμ. If one plots $\Delta\epsilon_M$ (−8.9) for the transfer of *N*-methylacetamide from 8 M urea to water and the values of $\Delta\epsilon_M$ (Glazer and Rosenheck, 1962) for the transfer of this compound from either dioxane (−73; n^{20}_{265} 1.4699) or cyclohexane (−44; n^{20}_{265} 1.4741) to water *vs.* the refractive indices at 265 mμ, an average coordinate for $\Delta\epsilon_M$ at n^{20} 1.50 is −165. Since the approximation of −30,000 has been made as the contribution from four tryptophyl equivalents to the experimentally observed value of −35,000 at 231 mμ, the remaining 5000 units could be accounted for by the transfer of only 30 peptide bonds (out of 239) from an environment with an effective refractive index of 1.50 at 289 mμ to the aqueous environment.

In the above discussion, possible contributions from tyrosine or phenylalanine (and other chromophores) have been ignored. Also, no attempt has been made to take into account differences in spectral peak positions of model compounds (with the exception of *N*-methylacetamide) and DIP-chymotrypsin. This could modify somewhat the values obtained for tryptophan contributions to the 231 mμ peak. In any case, and although conformation-dependent changes in peptide bond absorptivity may contribute, among other factors, to the difference peak at 231 mμ, it seems reasonable to conclude that environmental changes alone can represent a significant fraction of the spectral changes at this wavelength. Thus, one need not necessarily conclude, at least for DIP-chymotrypsin, that changes at the low-wavelength region are dominated by a helix–coil transition as suggested by Glazer and Rosenheck (1962).

Hamaguchi and Kurono (1963ab) have utilized data of the type exemplified by the final slopes as given in Figure 3 for an estimation of the tryptophyl equivalents exposed in the denatured state of muramidase. For example, a plot of $\Delta\epsilon_M$ at 292 mμ *vs.* 2-chloroethanol concentration showed an initial slope twice that of the final slope. They therefore concluded that the number of tryptophan residues exposed in the native protein was twice that of the protein at high solvent concentrations. Kronman *et al.* (1965) has determined the value of $\Delta\epsilon_M$ at 293 mμ/tryptophan equivalent exposed in the urea denaturation of α-lactalbumin using the experimentally obtained $\Delta\epsilon_M$ value and the red shift effect for tryptophan on transfer from water to 8 M urea. The magnitude of the denaturation blue shift experienced by RNAase in 8 M urea has also been calculated by Bigelow (1960) from red shift data for tyrosine and the experimental value of $\Delta\epsilon_M$ at 287 mμ. For at least this case, the additivity relationship assumed appears to hold since Bigelow (1964) has also obtained

equivalent results (−2600 *vs.* −2650) from data for the denaturation of RNAase in guanidinium chloride by extrapolation of the final slope in a $\Delta\epsilon_M$ *vs.* denaturant concentration plot to zero guanidinium chloride concentration. Bigelow also showed that the slope of this line was equal to the slope of the red shift effect upon comparison of oxidized RNAase in guanidinium chloride *vs.* oxidized RNAase in water.

Inspection of the data for the urea denaturation of DIP-chymotrypsin at 293 mμ (see Figure 3) shows that the final slopes cannot be used to calculate the number of tryptophan equivalents exposed in the denatured state. The data at, say, 8 M urea can also not be used in conjunction with the spectral perturbation of model compounds to determine the magnitude of the total blue shift due to exposure of buried tryptophans. As stated before (see Results), extrapolation of the final slopes at either pH 4 or 7 to zero urea concentration gave an ordinate value of −10,000. This and the fact that three (or essentially three) tryptophan equivalents are exposed in the native state at either pH 4 or 7 [results of this paper and of Williams *et al.* (1965)] make it obvious that different results will be obtained for the calculation of total tryptophan residues exposed (from the slopes) or for the magnitude of the blue shift depending on whether data at pH 4 or 7 are used. As examples, at pH 4 and 8 M urea, $\Delta\epsilon_M$ at 293 mμ is −7750. Perturbation of the spectrum of acetyl-D-tryptophanamide by urea gave a value for 7 × $\Delta\epsilon_M$ at 291.5 mμ of 4620 at 8 M. Using acetyl-L-tryptophan ethyl ester, the comparable value was 3885. Algebraic subtraction from $\Delta\epsilon_M$ for DIP-chymotrypsin gives either −12,170 or −11,635 for the total blue shift. Using the same model compound data (determined to be the same for acetyl-D-tryptophanamide at pH 7 as at 4) and the experimental value of −6800 at pH 7 and 8 M urea for DIP-chymotrypsin, one obtains −11,420 or −10,665 for the blue shift. Both sets of data are thus different from each other and larger than the value (−10,000) obtained by extrapolation of the slopes to zero urea concentration.

If the number of tryptophans exposed in the denatured state could be determined from the final slopes of the experimental data, then, for the case of DIP-chymotrypsin wherein the initial slope (pH 4 or 7) is 225 $\Delta\epsilon_M$ units-M^{-1} urea, the final slopes should be the same at both pH values and equal to 7(225/3) or 525 $\Delta\epsilon_M$ units-M^{-1} urea. The observed values are 332 at pH 4 and 413 at pH 7.

The above results thus serve to emphasize that, as a general procedure, it would seem that extrapolation of the final slope to zero denaturant concentration will yield at the ordinate intercept the most reliable value for the total blue shift. The slope of this line should also be equal to the slope of the line obtained by comparison of the absorbance of the denatured protein in the solvent used *vs.* the absorbance of the denatured protein in water. This criterion has been shown to be satisfied for the denaturation of RNAase in guanidinium chloride by Bigelow (1964).

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