ASSESSMENT OF CONFORMATIONAL CHANGES IN LOW-SULPHUR *S*-CARBOXYMETHYLKERATEINE FROM WOOL

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Summary

The effects of urea and guanidine hydrochloride on the ultraviolet absorption spectrum of the low-sulphur *S*-carboxymethylkerateine fraction of wool have been measured. In concentrated solutions of urea characteristic difference spectra were obtained with maxima of negative absorbance at 288, 280, and 240 mμ. The addition of guanidine hydrochloride or an increase in temperature gave similar negative difference maxima at the higher wavelengths. Calculation of the extent of unfolding of the protein chains from the difference in absorbance at all three maxima showed that the unfolding was 50% complete at a urea concentration of about 1·8M whereas a urea concentration of about 4·3M was required to decrease the helix content by 50%. Similar measurements on components 7 and 8, the two major constituents of SCMKA, showed that a 50% decrease in helix content was obtained with 2·8M and 0·8M urea respectively whereas the corresponding values for 50% unfolding assessed from difference spectral measurements were 2·2M and 1·2M urea respectively. It is suggested that the helical regions of components 7 and 8 aggregate specifically and that spectral measurements relate largely to non-helical portions of the chains.

I. INTRODUCTION

The ultraviolet absorption spectrum characteristic of the various chromophore groups of a protein in solution is known to be modified by changes in the solvent (Wetlaufer 1962). The incorporation of substances such as glycerol, sucrose, and other polyols in aqueous solutions of proteins causes a red shift in the spectrum which is usually attributed to physical changes in the properties of the solvent such as refractive index or dielectric constant or to solvent–solute interactions in the environment of the chromophores (Bigelow and Geschwind 1960; Leach and Scheraga 1960; Yanari and Bovey 1960). The magnitude of these effects has been used to assess the number of chromophore groups exposed to the solvent and has been found to increase considerably when proteins are denatured (Herskovits and Laskowski 1962; Williams, Herskovits, and Laskowski 1965). Reagents such as urea or guanidine hydrochloride, though producing a red shift at low concentrations, produce a blue shift at concentrations sufficient to denature the proteins (Martin and Bhatnagar 1966) and the magnitude of this effect has been used as a measure of the extent of unfolding of the protein chains.

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In order to extract a high proportion of wool in the form of soluble protein derivatives it is necessary to rupture most of the disulphide bonds in the fibre. This may involve considerable changes in conformation of the protein but nevertheless it has been shown from optical rotatory dispersion measurements that the low-sulphur fraction of *S*-carboxymethylkerateine from wool (SCMKA) is 50–60% helical in solution at pH 9 (Harrap 1963). The two major components of SCMKA (designated components 7 and 8) have been isolated by various techniques (O'Donnell and Thompson 1964; Thompson and O'Donnell 1965; Frater 1966; Dowling and Crewther, unpublished data) and shown to be partly helical also. In the presence of 8M urea, 5M guanidine hydrochloride, or 8M LiBr, SCMKA undergoes a reversible conversion to a random conformation and similar changes occur on raising the temperature from 25 to 80°C (Harrap 1963).

Since difference spectral measurements at a wavelength of about 280 mμ relate to the tyrosine residues and hence chiefly to non-helical portions of the protein chains (Crewther and Harrap 1967) whereas optical rotatory dispersion measurements reflect changes in helical or similarly ordered structures, the present study has been made to compare the effects of protein denaturants on SCMKA and components 7 and 8 by means of these techniques.

II. Materials and Methods

Wool top was washed with light petroleum, ethanol, and distilled water and air-dried. High-sulphur protein was preferentially extracted from 50 g of wool by treating with 0·8M potassium thioglycollate at pH 10·3 and 2°C for 18 hr. The low-sulphur protein fraction was released from the residue by immersion in cold water and the extracted material alkylated with sodium iodoacetate at pH 9·0 as described by Harrap and Gillespie (1963). The *S*-carboxymethyl derivative of the low-sulphur protein (SCMKA) was dialysed against deionized water and lyophilized. The two main constituents of SCMKA, components 7 and 8, were prepared by fractional precipitation of SCMKA (Dowling and Crewther, unpublished data).

Urea (Analar), guanidine hydrochloride, and 2-chloroethanol were B.D.H. products and were purified prior to use. Urea was recrystallized as described by Martin and Frazier (1965). 2-Chloroethanol was purified and distilled according to Goldstein and Katchalski (1960). Guanidine hydrochloride was recrystallized twice from methanol. Deionized water was used throughout. Solutions of urea, guanidine hydrochloride, and protein were filtered through Millipore filters before making spectral measurements.

The ultraviolet difference spectral measurements in the 340–230 mμ region were obtained using a Beckman model DK-2 recording spectrophotometer. An aqueous protein solution of the same pH and concentration per unit volume was used as a reference for the difference spectra at the various concentrations of urea, guanidine hydrochloride, or 2-chloroethanol. Matched cuvettes (1 cm) were used in all the experiments. Difference spectra, in general, have been corrected for solvent absorption by a separate determination of the appropriate correction curve. In temperature studies a special cuvette holder was designed to maintain the reference

cuvette at a constant temperature by circulating water from a thermostat-controlled bath while varying the temperature of the sample cuvette chamber using a similar bath. Sufficient time was allowed for equilibration at each temperature.

Unless otherwise stated protein concentration was determined by measurement of refractive index increment at 546 mμ using a Phoenix differential refractometer. The specific refractive index increment, dn/dc, was taken to be $1{\cdot}90\times10^{-3}$ for SCMKA. In most experiments measurements of protein concentration were confirmed by determining the absorbance of the solutions at 278 mμ, having first determined the extinction coefficient, $E^{1\%}_{1\,cm}$, of the particular SCMKA preparation at this wavelength. This value was obtained by measuring the absorbance of solutions prepared from the SCMKA and determining the moisture content of the SCMKA by drying *in vacuo* at 40°C.

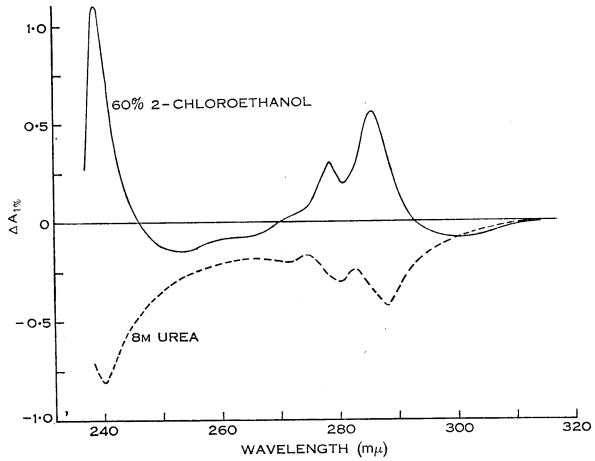

Fig. 1.—Difference absorption spectra of SCMKA at pH 8·0 in 8M urea or in 60% (v/v) 2-chloroethanol *v.* SCMKA in water at the same concentration and pH.

In general solutions containing 1·5 mg protein per millilitre were used in the difference spectral measurements, as Beer's law holds good at this concentration. The data are presented as difference absorbance (ΔA) calculated for a 1% solution (w/v). All measurements were made at pH 8·0, solutions being adjusted with dilute KOH before making up to volume. Measurement of pH was by means of a glass-electrode assembly and potentiometer. The temperature of all solutions was maintained at 30°C unless otherwise indicated.

Optical rotatory dispersion measurements were made at wavelengths of 578, 546, 436, 405, and 365 mμ using a Perkin–Elmer Model 141 spectropolarimeter. Specific rotation data as a function of wavelength were plotted in terms of the Moffit–Yang equation (1965) to obtain the parameter b_0, taking $\lambda_0 = 212$ mμ. Helix content was calculated from the expression:

$$\text{Helix content (\%)} = -100\, b_0/630.$$

III. Results

(a) *Spectral Changes in Various Solution Environments*

Difference spectra for solutions of SCMKA obtained by incorporating 8M urea or 60% (v/v) 2-chloroethanol in the solutions at pH8 without added salt are presented in Figure 1. Pronounced negative maxima occurred at 288, 280, and 240 mμ in 8M urea while a spectrum characterized by positive peaks with maxima at 286, 278, and 238 mμ was obtained from a comparison of SCMKA in water and in 60% 2-chloroethanol. Similar positive maxima of different magnitudes were obtained in various concentrations of 2-chloroethanol. Spectral changes were complete in less than 2 min, no further changes taking place over a period of 4 hr. The greater absorbance of the protein in 2-chloroethanol compared with the reference solvent would be expected on the basis of the higher refractive index of the aqueous 2-chloroethanol.

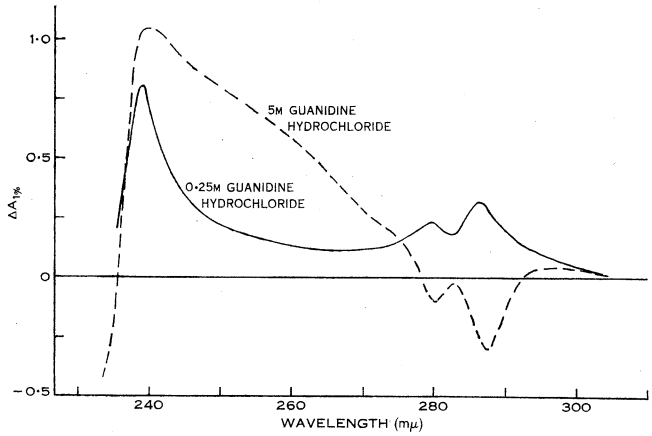

Fig. 2.—Difference absorption spectra of SCMKA in 0·25M and 5M guanidine hydrochloride *v.* SCMKA in water at the same concentration and pH.

Exposure of SCMKA to various concentrations of guanidine hydrochloride resulted in the difference spectra shown in Figure 2. At a concentration of 0·25M the peaks generated at 286, 279, and 238 mμ show positive maxima. A positive to negative transition occurred in the high wavelength region with increase in concentration of the denaturant, but the peak at 238–240 mμ remained positive throughout.

(b) *Effects of Increase in the Concentration of Urea on Spectrum*

Figure 3(*a*) shows the relationship between the concentration of urea in solutions of SCMKA and $\Delta A_{1\%}$ at wavelengths of 288 and 280 mμ. At concentrations up to 1M there is evidence of a solvent-induced red shift but with further increase in urea concentration this solvent effect is obliterated by an opposite trend, $\Delta A_{1\%}$ reaching a minimum at a urea concentration of about 5M. At higher concentrations there is little further red shift due to solvent effects. The change from a red shift to a blue

shift resembles closely the effects observed when a globular protein is converted from a native to an unfolded state. Hence the red shift at low concentrations of urea may be attributed to perturbation of the spectrum due to exposed tyrosyl residues and the blue shift at higher concentrations to the rupture of existing tyrosyl interactions in an ordered portion of the molecule. Calculation of the fractions of the initially inaccessible tyrosyl residues made accessible to the solvent (Martin and Bhatnagar 1966) at various urea concentrations was carried out on the assumption that all tyrosine residues were exposed to the solvent when $\Delta A_{1\%}$ reached its minimum value (Fig. 3). Figure 4 shows that the unfolding process had reached 50% completion at a urea concentration of 1·8M. Parallel changes occurred in the peaks at 240, 280, and 288 mμ.

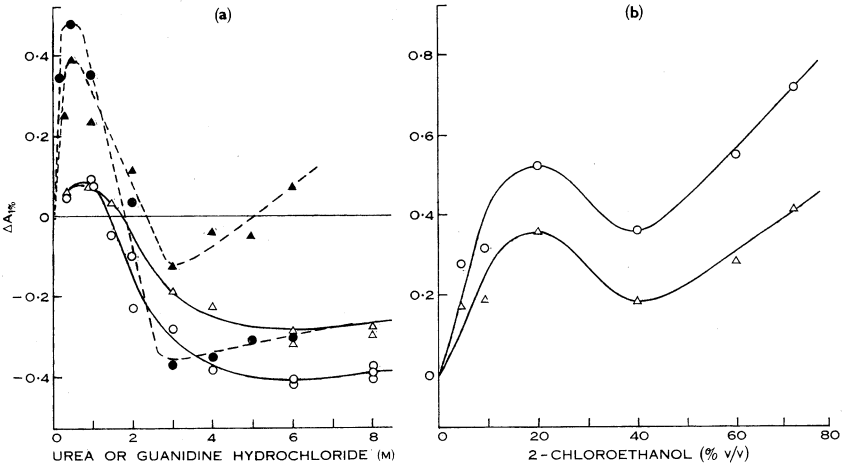

Fig. 3.—(*a*) Variation in difference absorbance at 286–288 mμ (○, ●) and 278–280 mμ (△, ▲) with increase in concentrations of urea (open symbols) or guanidine hydrochloride (closed symbols). (*b*) Variation in difference absorbance at 286 mμ (○) and 278 mμ (△) with increase in concentration of 2-chloroethanol.

No assessment has been made of the number of tyrosyl residues accessible to the solvent in aqueous solution because the solutions of SCMKA are known to contain at least two major components together with small amounts of several other proteins many of which are rich in tyrosine, phenylalanine, and tryptophan. It seemed possible that these minor components in the solution may contribute a major part to $\Delta A_{1\%}$. Harrap (unpublished data) has shown that most of the fraction rich in tyrosine can be removed from SCMKA by passing through Sephadex G-200 in 0·05M sodium tetraborate solution and collecting the main peak eluted before the minor components. The material obtained by this procedure shows an increase of 50–70 in the value of $-b_0$ calculated from optical rotatory dispersion measurements using the equation of Moffitt and Yang (1956). When a purified sample of SCMKA prepared in this way was used to obtain difference spectra in the presence of urea, the results obtained were almost identical with those recorded in Figures 1, 3, and 4. Hence the major components of SCMKA are probably responsible for the observed difference spectra.

Harrap (1963) has shown also that the b_0 value of about -320 obtained for SCMKA at pH 9·1 is almost unaffected by urea concentrations up to 2·0M but approaches zero as the concentration of urea is increased to 8M. The $-b_0$ value was decreased to about one-half its original value by a concentration of about 4M. In order to compare the results obtained from difference spectra with those obtained from optical rotatory dispersion Harrap's experiments with SCMKA in urea solutions were repeated with the purified SCMKA used in the preceding experiment at pH values of 9·1 and 8·0. At the former pH value the results were identical within experimental error with those of Harrap, a concentration of about 4M urea being required to

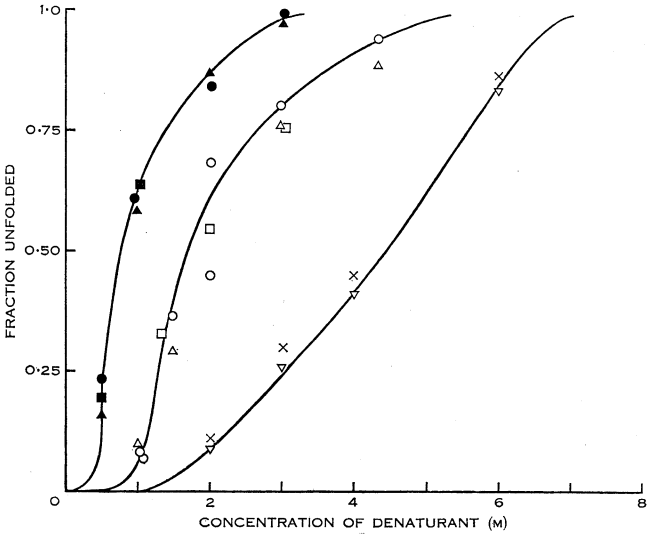

Fig. 4.—Relationship between fraction of SCMKA in the unfolded state and concentration of denaturants calculated from difference spectra using urea (○, □, △) and guanidine hydrochloride (●, ■, ▲) as denaturants and from optical rotatory dispersion measurements using urea at pH 8·0 (▽) and 9·1 (×) as denaturant. Spectral measurements at 286–288 (○, ●), 278–280 (△, ▲), and 238–240 (□, ■) mμ.

give a value for $-b_0$ one-half that of SCMKA in buffer solution (Fig. 4). At pH 8·0 the protein was slightly more stable, as would be expected as pH values approached the isoionic point, and a urea concentration of about 4·3M was required to decrease the $-b_0$ value by one-half.

(*c*) *Effects of Increase in the Concentration of Guanidine Hydrochloride on Spectrum*

Figure 3(*a*) shows that, like urea, guanidine hydrochloride had an immediate solvent effect on exposed chromophores in SCMKA causing an increase in $\Delta A_{1\%}$ up to concentrations of 0·05M. With further increase in guanidine concentration the protein underwent a transition resulting in a decrease in $\Delta A_{1\%}$ to a minimum value in the negative range at a concentration of about 3M. With further increase in guanidine concentration the values of $\Delta A_{1\%}$ again increased. Calculation of the fraction of inaccessible tyrosyl residues made accessible to the solvent (Fig. 4) showed that a concentration of about 0·8M guanidine hydrochloride was required to make 50% of these groups accessible.

Although at concentrations less than 5M guanidine hydrochloride caused more extensive changes in absorbance than did urea at equal concentrations, the complete transition (>3M guanidine hydrochloride, >5M urea) involved approximately the same change in $\Delta A_{1\%}$ in each solvent after correction for solvent effects. This correction was made by extrapolating the curves above and below the concentration range corresponding with the transition to the unfolded state to zero concentration (Martin and Bhatnagar 1966). The corrected values of $\Delta A_{1\%}$ obtained in this way were about 0·450 at 286–288 mμ and 0·375 at 278–280 mμ for both urea and guanidine

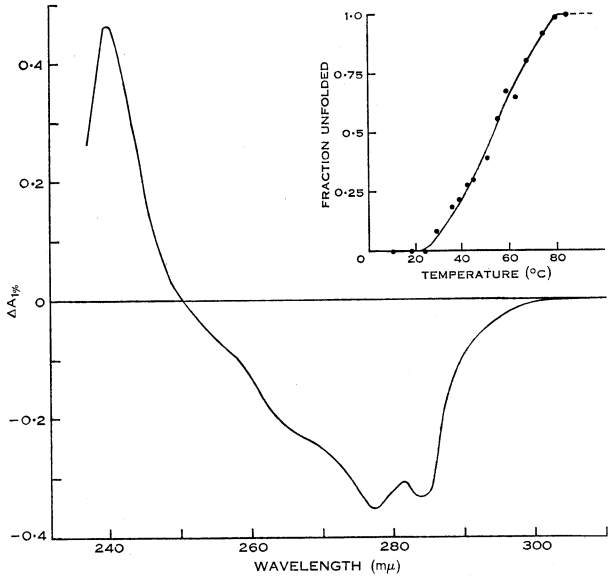

Fig. 5.—Difference absorption spectrum of SCMKA at high temperature *v.* low temperature (14°C) in aqueous solution at pH 8·0. Insert: fraction of SCMKA in the unfolded state as a function of temperature calculated from difference absorbance at 277 mμ.

hydrochloride. Although the peak at about 240 mμ remained positive over the whole range of guanidine concentration, a major transition was apparent between 0·5 and 3·0M with decreasing values of $\Delta A_{1\%}$ (Fig. 3). Apparently the very large solvent effect at this wavelength is sufficient to maintain positive values of $\Delta A_{1\%}$.

(*d*) *Effects of Increase in the Concentration of 2-Chloroethanol on Spectrum*

In 2-chloroethanol, a helix-favouring solvent, the band displacement to the red increases with increasing refractive index of the solvent. The perturbation effect of this solvent on the exposed chromophores at various concentrations of 2-chloroethanol is shown in Figure 3(*b*). The red shift was maximal at about 20% and as the concentration was increased, a transition of small magnitude occurred, but the absorbance remained positive. The solvent effect became predominant again at concentrations greater than 40% 2-chloroethanol.

(e) *Thermal Transition of SCMKA*

The temperature difference spectrum of SCMKA (Fig. 5) obtained by comparing the absorbance at 60°C with that at 14°C is characterized by peaks of negative absorbance at 277 and 284 mμ which increase in magnitude with increasing temperature. The absorbance differences at 277 mμ for a range of temperatures were used to calculate the fraction of the protein converted to the conformation favoured by the higher temperatures making the assumption that this change was complete at a temperature of 80°C. The insert in Figure 5 shows that the transition occurs over the temperature range 25–80°C with 50% conversion at about 54°C.

TABLE 1

UREA CONCENTRATION PRODUCING 50% UNFOLDING OF LOW-SULPHUR PROTEINS AS ASSESSED BY OPTICAL ROTATORY DISPERSION AND DIFFERENCE SPECTRAL MEASUREMENTS

Protein Fraction	Urea Concentration (M) Causing 50% Unfolding	
	Optical Rotatory Dispersion	Difference Spectra
SCMKA	4·3	1·8
Component 7	2·8	2·2
Component 8	0·8	1·2

(f) *Effects of Urea on Components 7 and 8*

Difference spectral measurements on solutions of components 7 and 8 containing various concentrations of urea provided data similar to that relating to SCMKA recorded in Figures 3 and 4. The curves obtained for components 7 and 8 were similar in shape to those obtained for SCMKA but quantitatively different. Table 1 lists the urea concentrations required to produce 50% unfolding of the protein fractions as measured by the magnitude of the difference spectrum at 287 and 276 mμ.

Optical rotatory dispersion measurements show greater differences in the effects of urea on the conformation of these protein fractions (Fig. 6; Table 1). It is apparent from the curves in Figure 6 that mixtures of components 7 and 8 contain considerably more helical material than solutions of the separate components particularly in the presence of urea at concentrations greater than 2M.

IV. DISCUSSION

The effects of urea and similar protein denaturants on the conformation of proteins are too complex and the mechanism of their effects too uncertain to permit conclusions regarding the exact location of tyrosine or other chromophore groups in the molecules or molecular aggregates of SCMKA. Nor has any attempt been made to estimate the numbers of solvent-accessible and solvent-inaccessible chromophore groups in the protein molecules as the solutions are known to contain several

molecular species with uncertain molecular weights. Tryptophan residues contribute little if anything to the difference spectra observed in view of the absence of peaks in the difference spectra at about 293 mμ, tyrosine residues being chiefly responsible for the results obtained at 277–288 mμ. It seems reasonable to assume that the blue shift observed at 277–288 mμ at high concentrations of urea or guanidine hydrochloride and at high temperatures results from the unfolding of the protein chains and the consequent exposure of "buried" tyrosine residues to the solvent as, for example, Bigelow and Geschwind (1960) have suggested. The blue shifts are

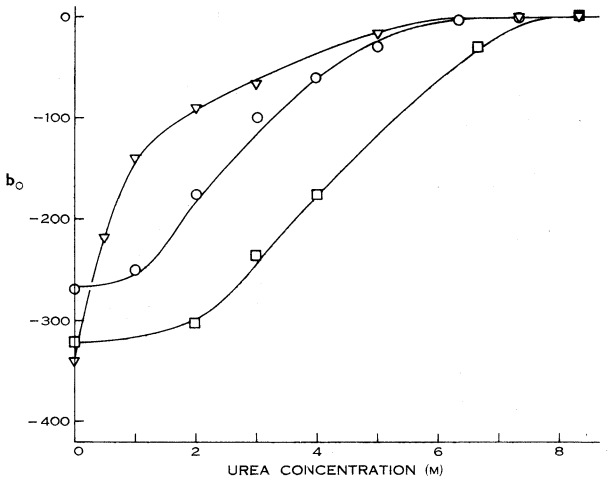

Fig. 6.—Relationship between urea concentration and b_0 value for SCMKA (□), component 7 (○), and component 8 (▽) at pH 8·0.

superimposed on a background of increasing red shift of the absorption bands which probably results from changes in refractive index and other properties of the solvent and in the interaction of solvent and chromophores. With solutions containing additives such as guanidine hydrochloride the red shift is so marked that $\Delta A_{1\%}$ remains positive even when a transition occurs to the unfolded state. Such a transition appears as a section with a negative slope in the curve relating $\Delta A_{1\%}$ and the concentration of the additive [Fig. 3(*a*)]. Since such a section with negative slope occurs in the curve relating $\Delta A_{1\%}$ with the concentration of 2-chloroethanol [Fig. 3(*b*)], and as 2-chloroethanol is known to increase the helix content of solutions of SCMKA as measured by optical rotatory dispersion techniques (Harrap 1963), it is possible that this increase in helicity is accompanied by the rupture of other interactions within the protein chains involving tyrosine residues. On the other hand the tyrosine residues may occur in non-helical regions of the chains which could conceivably be unfolded by 2-chloroethanol even though the helicity of other portions of the chains was increased.

More definite evidence that the helicity of the chains of SCMKA cannot be taken as the sole index of the conformation of these proteins is provided by a comparison of the effects of urea on the values of b_0 obtained by applying the Moffitt-

Yang (1956) equation to optical rotatory dispersion data and on $\Delta A_{1\%}$ at wavelengths of 240, 280, and 288 mμ (Figs. 1, 3, and 4). The unfolding of the protein by urea, as indicated by the difference spectra is 90% complete at a urea concentration of about 4·3M at pH 8·0 whereas under similar conditions the helix content is decreased by only 50%. On the other hand the effects of temperature on $\Delta A_{1\%}$ and on helix content are quantitatively similar (cf. Harrap 1963; Fig. 5).

Comparison of the properties of components 7 and 8 with those of SCMKA has shown that the helical regions of components 7 and 8 are destroyed by urea at much lower concentrations than those required to produce a similar effect on SCMKA (Fig. 6) whereas differences in the effects of urea on the difference spectra of the three preparations at wavelengths of about 280 mμ are less marked and not in the same relative order (Table 1). This shows further that the effects of urea on the accessibility of tyrosine residues to the solvent are independent of the degree of unfolding of the helical portions of the molecules. This result might be expected from the relatively low tyrosine content of helix-rich fractions obtained from SCMKA by partial proteolysis (Crewther and Harrap 1967) and it seems probable that the difference spectral measurements in urea relate chiefly to non-helical portions of the molecules. The data also support the evidence of Dowling and Crewther (unpublished data) that the helical portions of components 7 and 8 are stabilized by a specific interaction between the two components in a molar ratio of component 7 to component 8 of 2 : 1. The present data suggest that the non-helical portions of the chains are not stabilized by this interaction since the urea concentration causing 50% unfolding of the portions of SCMKA rich in tyrosine is the weighted mean of the urea concentrations having similar effects on components 7 and 8 separately (Table 1). It is assumed for this calculation that these components are present in a molar ratio of 2 : 1 in SCMKA (Thompson and O'Donnell 1964).

V. Acknowledgment

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VI. References

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