# Conformation of Cytochromes. III. Effect of Urea, Temperature, Extrinsic Ligands, and pH Variation on the Conformation of Horse Heart Ferricytochrome *c**

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ABSTRACT: The circular dichroic spectra of horse heart ferricytochrome *c* as a function of increasing urea concentrations, rising temperatures, variation of pH, and in the presence of extrinsic ligands, imidazole, cyanide, and azide, have been examined over the wavelength range 200–450 mμ. The denaturation of ferricytochrome *c*, whether induced by urea or by temperature elevation, occurs in two distinct thermodynamic steps. The first step of urea denaturation, centered at about the 3 M concentration, seems to be exclusively the uncoupling of the polypeptide chain from close proximity to the heme group, whereas the corresponding thermal step, centered at about 53°, is a composite of both the uncoupling and the partial unfolding of the polypeptide chain. The second thermal denaturation step centered at 82° is predominantly the helix–coil transition, whereas the corresponding urea step, centered at 6.5 M concentration, seems to involve, in addition to the helix–coil transition, the disruption of the protein–heme bonds, possibly by replacement of intrinsic ligand by urea molecule. The role of the protein–heme bonds in stabilization of the protein and thereby the dissymmetry of the heme group and the coupling of the helix–heme transitions is suggested.

The addition of extrinsic ligands, the alteration of pH between 3 and 10, and the addition of urea up to about 4.5 M concentration result in almost identical alterations of the circular dichroism spectrum, both in the intrinsic absorption region, sensitive to polypeptide conformation, and in the Soret absorption region, representing the dissymmetric environment of the heme group. The ellipticity in the intrinsic absorption region increases, reflecting an increase in the rotatory strength of the amide transitions in an α-helical conformation, whereas in the Soret region the contributions from three of the five dichroic bands are either eliminated or reduced. This reciprocal behavior of the rotatory strengths is consistent with the expected behavior of transitions deriving their optical activity from electric–electric dipole coupling. Thus the conclusion that the conformation of cytochromes involves extensive coupling of protein–heme transitions is supported. Two of the four ellipticity bands in the aromatic absorption region, the 282- and 288-mμ ellipticity bands, seem to arise from the single invariant tryptophan residue; the band centered around 251 mμ possibly reflects contributions from tyrosine side chains, and the 262-mμ band appears to be associated with the heme group. The 282- and 288-mμ ellipticity bands show relatively no sensitivity to alterations of interplay between protein and prosthetic group, induced either by replacement of intrinsic ligands by extrinsic ligands or by variation of pH, or by initial thermal denaturation, whereas both are either eliminated or appreciably reduced by the unfolding of the polypeptide chain. The 251- and the 262-mμ ellipticity bands, on the other hand, show sensitivity to both the alteration of the interplay as well as the unfolding of the polypeptide chain.

The optical rotatory dispersion studies of horse heart cytochrome *c*[1] thus far undertaken (Ulmer, 1965; Urry and Doty, 1965; Myer and Harbury, 1966; Mirsky and George, 1966) indicate that the change of the valence state of heme iron induces significant alteration in the asymmetric environment of the prosthetic group, the aromatic chromophores, and possibly in the conformation of the polypeptide chain as well. The regeneration of the near-native dispersion curves upon reduction of a solution containing denaturing agent (Urry, 1965) and the presence of a two-step thermal transition (Urry, 1965; Myer *et al.*, 1966) have been interpreted as a reflection of the significant role ascribed to the interplay between the protein and the prosthetic group. The simplification of the optical rotatory dispersion curves upon addition of extrinsic ligands, by pH variation (Myer and Harbury, 1965; Ulmer, 1966), by lowering of ionic strength at acid pH (Myer *et al.*, 1966; Mirsky and George, 1966), and by esterification of the protein (Myer *et al.*, 1966) reflects the significance of both the nature of the central coordinated complex as well as the charge distribution of the molecule. The complexity of the optical rotatory dispersion curves, however,

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[1] Abbreviation used: cytochrome *c*, refers to horse heart ferricytochrome *c*.

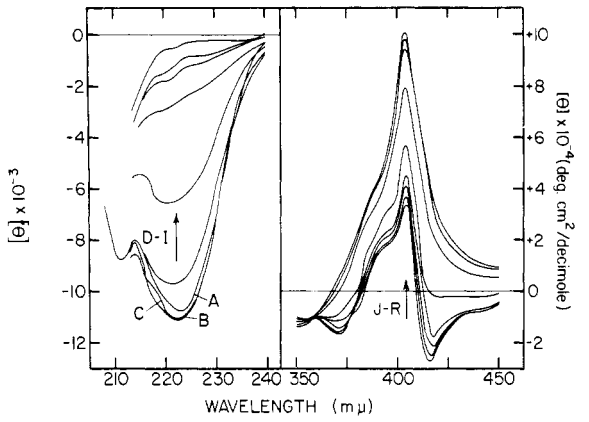


FIGURE 1: Circular dichroism spectra of horse heart ferricytochrome *c* as a function of urea concentration. Potassium phosphate buffer (0.1 M, pH 6.8); temperature, 25°. (A) No urea; (B) 2 M; (C) 4 M; (D) 6 M; (E) 7 M; (F) 8 M; (G) 8.5 M; (H) 9 M; (I) 9.5 M; (J) no urea; (K) 3 M; (L) 4 M; (M) 5 M; (N) 6 M; (O) 7 M; (P) 8 M; (Q) 9 M; and (R) 9.5 M.

does not permit specific analysis of the alterations encountered during various modes of perturbation, at least in light of the alterations and the origins of the rotatory strengths of various transitions. Such a situation is inherent in a system with multiple overlapping Cotton effects, which is further complicated by the property of a Cotton effect to possess limbs of opposite sign on each side of the transition and to extend rotatory contributions over the entire spectral region. Dichroic absorption spectroscopy, a phenomenological equivalent of optical rotatory dispersion, on the other hand, has the advantage of presenting discrete bands corresponding to each of the active transitions, which are of single sign (either positive or negative) and extend contributions over only a narrow wavelength region. The circular dichroic spectrum of horse heart cytochrome *c* (Y. P. Myer, unpublished data) is exceedingly rich in details over the entire spectral region, 188–600 mμ, reflecting the contributions from transitions arising from various chromophores. Since the origins of most of these transitions, especially in the intrinsic and the Soret absorption regions, are well characterized, the investigations of alterations of their rotatory strengths, a parameter directly proportional to the height of each band, would aid in interpretation and thus understanding of the conformational implications for the molecule. In addition to other studies on these lines, we have been investigating the circular dichroism spectra of various cytochromes *c* under widely different conditions. A broad comparison of such results for horse heart ferricytochrome *c* is presented.

## Materials and Methods

Horse heart cytochrome *c* (type III) was purchased from Sigma Chemical Co. The preparation was used without any further purification, since the comparison of optical rotatory dispersion data with those obtained with a crystalline sample did not show any variations of significance (Myer and Harbury, 1965). Ultra Pure urea (lot R3280, Mann Research, Inc.), imidazole (95B-1170, Sigma Chemical Co.), potassium cyanide, sodium azide, and all other chemicals employed were of analytical grade.

The circular dichroism and optical rotatory dispersion measurements were carried out on a JASCO/ORD/CD/UV-5 spectropolarimeter. The slit width of the instrument was programmed to yield constant light intensity throughout the spectral region. A constant slit width of 2 mm was maintained during investigation below 240 mμ. Cylindrical cells with fused-quartz windows and varying in path lengths from 0.1 to 50 mm were used. A water-jacketed, 10-mm cell with fused-quartz windows was employed during the investigation of the effect of temperature variation. All other investigations were conducted at room temperature. The concentration of the protein was determined spectrophotometrically using a molar absorptivity value of $1.12 \times 10^4$ l. $M^{-1}$ at 528 mμ. The rotations are expressed as mean residue rotation, [*m*], in the intrinsic absorption region, and as molar rotations, [M], in the other parts of the spectrum. The uncertainty of the measurements at the 231-mμ trough is ±8% and at the 410-mμ peak of the order of ±10%. The ellipticities were calculated from the expression, $[\theta] = 2.303(4500/\pi)(\epsilon_L - \epsilon_R)$. The ellipticities below 240 mμ are expressed as mean residue molecular ellipticities, degrees $cm^2$ per decimole of amino acid residues, and those above 240 mμ are molecular ellipticities, degrees $cm^2$ per decimole of protein and are of precision better than ±5% over the entire spectral region investigated.

Since commercially available cytochrome *c* is always in equilibrium with a certain fraction of ferrocytochrome *c*, it was necessary to ensure complete oxidation of samples. A solution containing potassium ferricyanide, amounting to about 10% of the total protein contents, was added to samples prior to both the spectrophotometric and circular dichroism measurements. Phosphate–KOH buffer (0.1 M, pH 7.0) was employed unless otherwise stated in the text. The changes of pH were made by addition of concentrated hydrochloric acid or 6 M KOH, and no correction in concentration of the solution due to dilution was made. The pH measurements were made on a Leeds-Northrup pH meter, type 7403 with expanded scale. Corrections due to the alteration of the refractive index were not made.

## Results

The effect of increasing concentrations of urea on the circular dichroism and the optical rotatory dispersion spectra of horse heart ferricytochrome *c* is shown in Figures 1 and 2, and in Figure 3 are shown the dichroic curves as a function of temperature. The

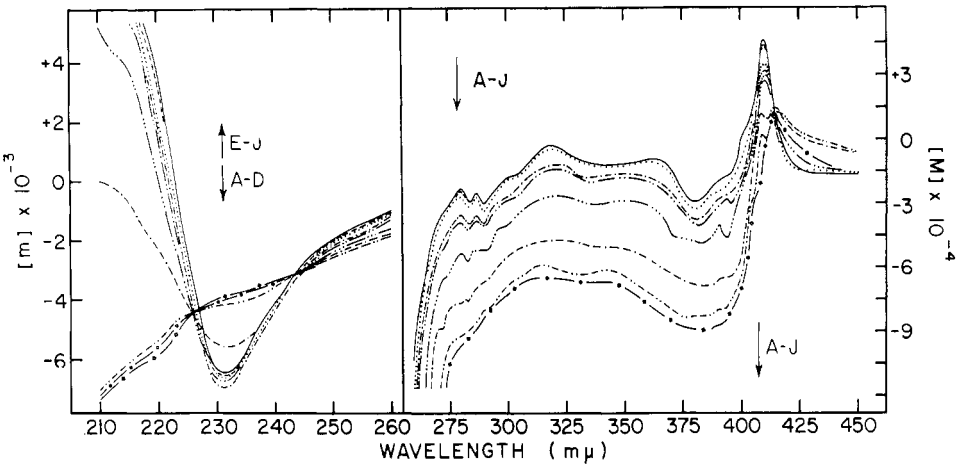


FIGURE 2: Optical rotatory dispersion of horse heart ferricytochrome *c* as a function of urea concentration. Potassium phosphate buffer (0.1 M, pH 6.8); temperature 25°. (A) ——, no urea; (B)– – –, 2 M; (C)· · · ·, 3 M; (D) – · – · –, 4 M; (E) – · · · · · –, 5 M; (F) — · · · — · · · —, 6 M; (G)– – · – · – · – –, 7 M; (H) – – · · · – –, 8 M; (I) –O–O–, 9 M; and (J) –◊–◊–, 9.5 M.

effect of temperature on the optical rotatory dispersion has been reported earlier (Myer *et al.*, 1966; Urry, 1965). As expected, the increasing urea concentrations and the rising temperatures induce alterations resulting in significant simplification of both the circular dichroism and the optical rotatory dispersion curves. With increasing urea concentration, the ellipticity at 222 mμ and the levorotation at the extremum near 231 mμ, the extrema characteristic of helical proteins, first increase and subsequently decrease to values comparable to those seen in random polypeptides[2] (Holzwarth and Doty, 1965; Simmons *et al.*, 1961). Rising temperatures, on the other hand, cause a continuous decrease of the dichroic absorption at 222 mμ as well as of the rotation at the intrinsic minimum (Myer *et al.*, 1966), a change similar to that seen upon helix–coil transition in simple polypeptide systems (Holzwarth and Doty, 1965; Simmons *et al.*, 1961). However, the values at these wavelengths at the highest temperature thus far investigated (98°) are considerably larger than can be accounted for on the bases of contributions from random model polypeptides (Holzwarth and Doty, 1965). The relatively large residual ellipticity with a well-defined shoulder at about 222 mμ therefore seems to suggest that only partial unfolding of the polypeptide chain has occurred during the thermal denaturation of the protein.

Paralleling the alteration in the intrinsic absorption region, the complex dichroic patterns over the entire spectral region are remarkably simplified, especially in the Soret and the aromatic absorption regions. In the complex Soret dichroic pattern, the increasing concentrations of urea seem to eliminate the contributions from transitions generating the 440-, 417-, and the 372-mμ negative minima; rising temperatures result in elimination of contributions to the two negative dichroic peaks (440 and 417 mμ) and possibly to the positive shoulder at about 390 mμ. The high ellipticity at 404 mμ with the crossover point at a lower wavelength for urea-denatured protein is attributable to the differential sensitivity of the 372- and 390-mμ peaks. These results thus suggest that the nature of alteration of the heme environment by urea must be different from that induced by elevation of temperature. This is further supported by the selective response of the transitions in the aromatic absorption region. The addition of urea obliterates the dichroic peaks at 288 and 282 mμ, and the double-peaked dichroic pattern in the wavelength region 250–260 mμ is replaced by an asymmetric dichroic band with a maximum at about 256 mμ of greater magnitude in comparison to both the 251- and 262-mμ dichroic peaks of the native protein (Y. P. Myer, unpublished data). The elevation of temperature up to the limit thus far investigated, 64°, a temperature considerably higher than the midtemperature of the first step of thermal transition (Figure 7), however, does not alter the ellipticity at 288 mμ and causes only a slight decrease in the magnitude of the 282-mμ peak; the 251- and 262-mμ bands are replaced by a single band of relatively small rotatory strength. It appears, therefore, that the nature of the alteration of the dissymmetric environment of the heme group and of the chromophores generating the dichroic bands in the aromatic absorption region, possibly the side-chain groups of tyrosine and tryptophan, when thermally induced, is significantly different from that inflicted by addition of urea. The latter possibly includes the effect of alteration of the chemical nature of the central coordinated

[2] It should be noted that even in the highest concentration of urea, the characteristic small positive peak at 218 mμ for random polypeptides (Holzwarth and Doty, 1965) is absent, possibly due to either small residual helical conformation or compensating dichroic contributions from transitions other than those associated with the amide bond.

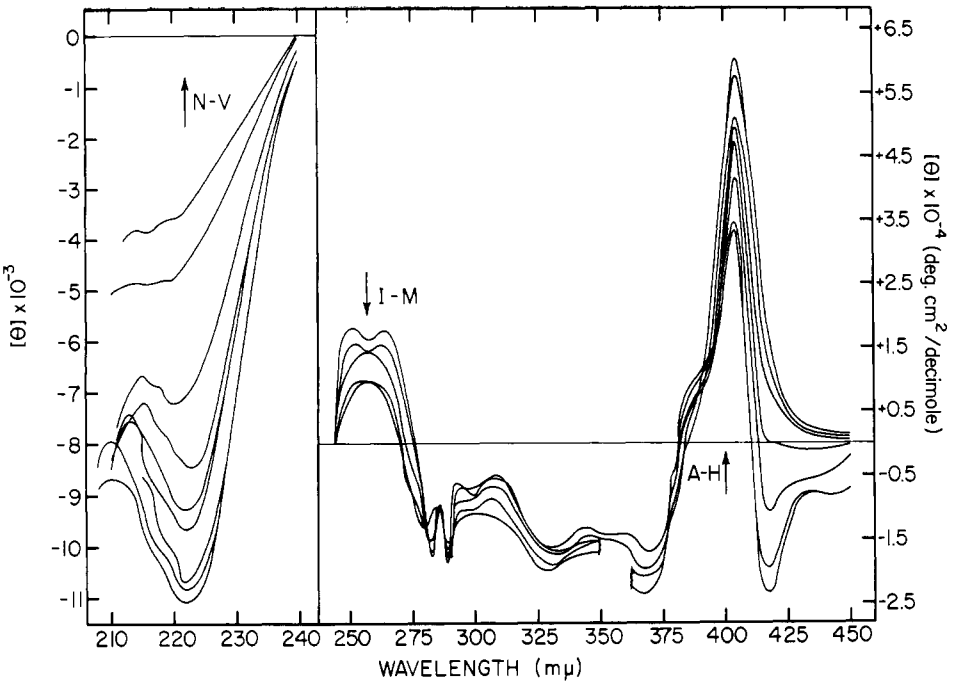


FIGURE 3: Circular dichroism spectra of horse heart ferricytochrome *c* as a function of temperature; pH 6.8 no buffer. (A) 25, (B) 36, (C) 48.5, (D) 60, (E) 69.5, (F) 79.5, (G) 90, (H) 98, (I) 25, (J) 35, (K) 45, (L) 54 (M) 64, (N) 28, (O) 40, (P) 50, (Q) 58, (R) 66, (S) 74, (T) 82, (U) 89, and (V) 98°.

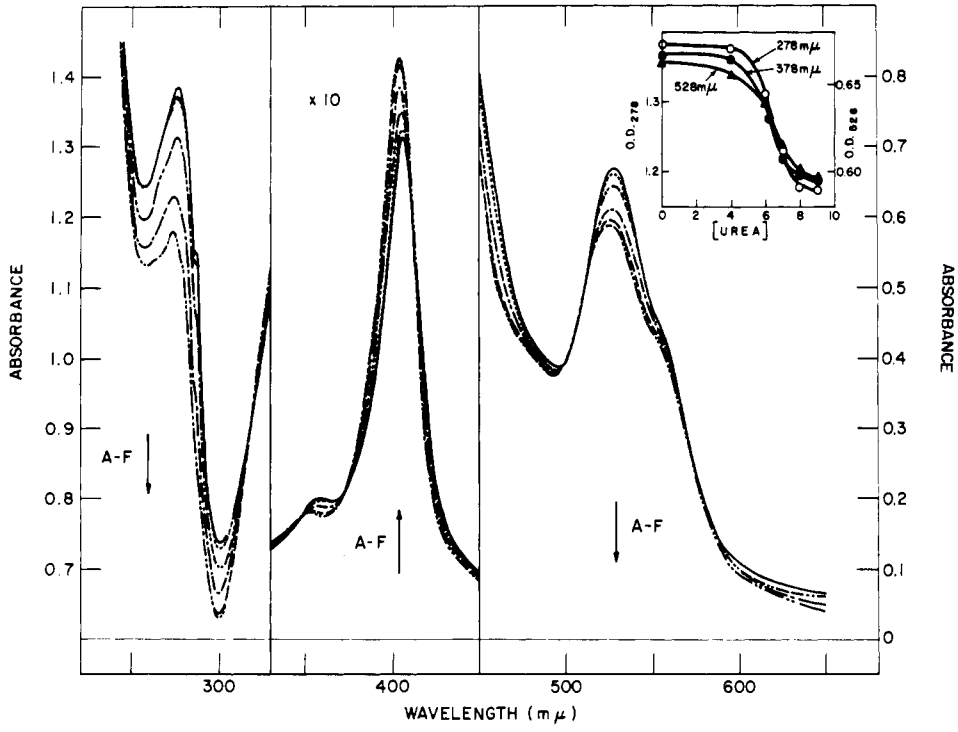


FIGURE 4: Absorption spectra of horse heart ferricytochome *c* as a function of urea concentration. Potassium phosphate buffer (0.1 M, pH 6.8); temperature, 25°. Soret region, *read ordinate on the right* × 10. (———) Buffer only; (· · · · ·) buffer and 4 M urea; (— · · — · · —) buffer and 6 M urea; (– · – · –) buffer and 7 M urea; (– – –) buffer and 8 M urea; (– · · – · · –) buffer and 9 M urea. Inset: Absorbance at 528, 378, and 278 mμ as a function of urea concentration; midpoint, 6.5 M. (○) 278, (●) 387, and (▲) 528 mμ.

complex (Stellwagen, 1967) in addition to the unfolding of the polypeptide chain.

In Figure 4 are shown the absorption spectra of horse heart ferricytochrome *c* as a function of urea concentration, and the 9.5 M urea spectrum is compared with the spectrum at 90° in Figure 5. As shown in the inset of Figure 4, the major alteration in the absorption spectrum seems to occur only toward higher urea concentrations. A blue shift of about 4 mμ in the Soret peak with a significant increase in the absorptivity indicates that increasing concentrations of urea induce alterations in the chemical nature of the central coordinated complex from that of a typical low-spin species to one of mixed-spin state, possibly by replacement of intrinsic ligand by urea nitrogen (Stellwagen, 1967). The elevation of temperature, on the other hand, does not induce any significant changes in the absorption spectrum (Figure 5) indicative of any noticeable alteration in the coordination of heme iron.

Figure 6A shows the changes in the ellipticities at 417, 404, 371, 288, and 222 mμ, and in Figure 6B are shown the alterations of rotations at 410, 381, 289, 279, and 231 mμ as a function of urea concentration. A similar plot for alterations from increasing temperatures is shown in Figure 7. It can be seen that irrespective of the nature of the transition, the denaturation of ferricytochrome *c* occurs in two steps. The first step of urea unfolding is centered at about the 3 M concentration and the second step at about the 6.5 M concentration. During thermal denaturation the first transition is centered at about 53° and the second at about 82°. As shown in the insets in Figures 6 and 7, and again irrespective of the nature of the transition, the two steps do show two distinct slopes, thus indicating that there exist two distinct thermodynamic processes. Also, the changes in the aromatic region, the Soret region, and the intrinsic absorption region during the second step of the urea-induced denaturation show identical slopes (Figure 6). The thermally induced alterations, both at 222 mμ and the Soret dichroic bands, exhibit identical slopes for each of the steps of the denaturation of the protein.

In Figures 8 and 9 is shown the effect of alteration of pH over the range 1.6–11.6, and in Figure 10 is shown the effect of ionic strength at extreme acid pH on the circular dichroism spectrum. Consistent with earlier observations (Myer and Harbury, 1965) based on optical rotatory dispersion studies, the circular dichroism pattern over the entire spectral region, especially the Soret region, shows remarkable sensitivity to hydrogen ion concentration. The complex circular dichroism curve remains unchanged in the narrow pH range of 5–8. Lowering of the pH to a value of 3 results in elimination of contributions to the 450-, 417-, and the 372-mμ peaks in the Soret region (resulting in dichroic patterns similar to those of urea-denatured ferricytochrome c); the dichroic absorption at 251 mμ seems to be obliterated and results in a concomitant increase in the ellipticities of the 222- and the 210-mμ negative peaks in the intrinsic absorption region. The dichroic peaks at 282 and 288 mμ, however,

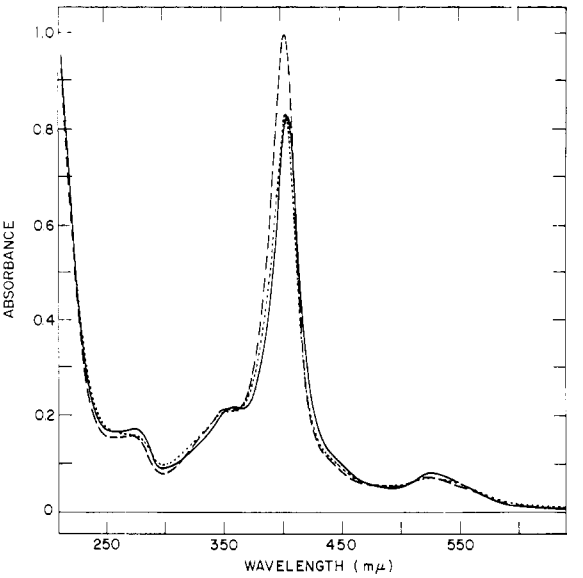


FIGURE 5: Absorption spectra of horse heart ferricytochrome *c*. (——) In phosphate–KOH buffer (0.1 M, pH 6.8), temperature, 25°; (– – –) in phosphate–KOH buffer (0.1 M) and 9.5 M urea (pH 6.8), temperature, 25°; (· · · · ·) in water (pH 6.8), temperature, 90°.

seem to be unaffected. Further lowering of pH induces changes which are predominantly centered in the aromatic and the intrinsic absorption regions. The rotatory strengths of the negative bands in the aromatic region are significantly reduced, and are accompanied by decrease of ellipticity at 222 mμ. The alterations to pH 3 therefore cause changes in the circular dichroism pattern which approach the first step of urea denaturation, whereas those encountered at lower pH values seem to involve partial unfolding of the polypeptide and possible alteration of the chemical nature of the central coordinated complex, and thus reflect a definite break in the trend of changes below pH 3.

If the pH of an unbuffered solution is brought to a value of 1.6, a significantly different type of dichroic pattern in both the Soret and the intrinsic absorption regions is encountered (Figure 10). The ellipticity of the major dichroic band in the Soret absorption region, the 404-mμ peak, decreases to about half the value as observed in a solution in 0.1 M phosphate–HCl at the same pH, with a shift to a smaller wavelength; the contributions at 282 and 288 mμ are obliterated, and the dichroic pattern in the intrinsic absorption region is replaced by one grossly similar to that of a random polypeptide (Holzwarth and Doty, 1965). The increase of the ionic strength of the solution by addition of KCl, however, results in regeneration of dichroic patterns which resemble those exhibited in 0.1 M phosphate–HCl buffer at this pH (Figure 10). From the foregoing, it is evident that addition of chloride ion affects not only the conformation of the polypeptide chain, but also that of the heme group

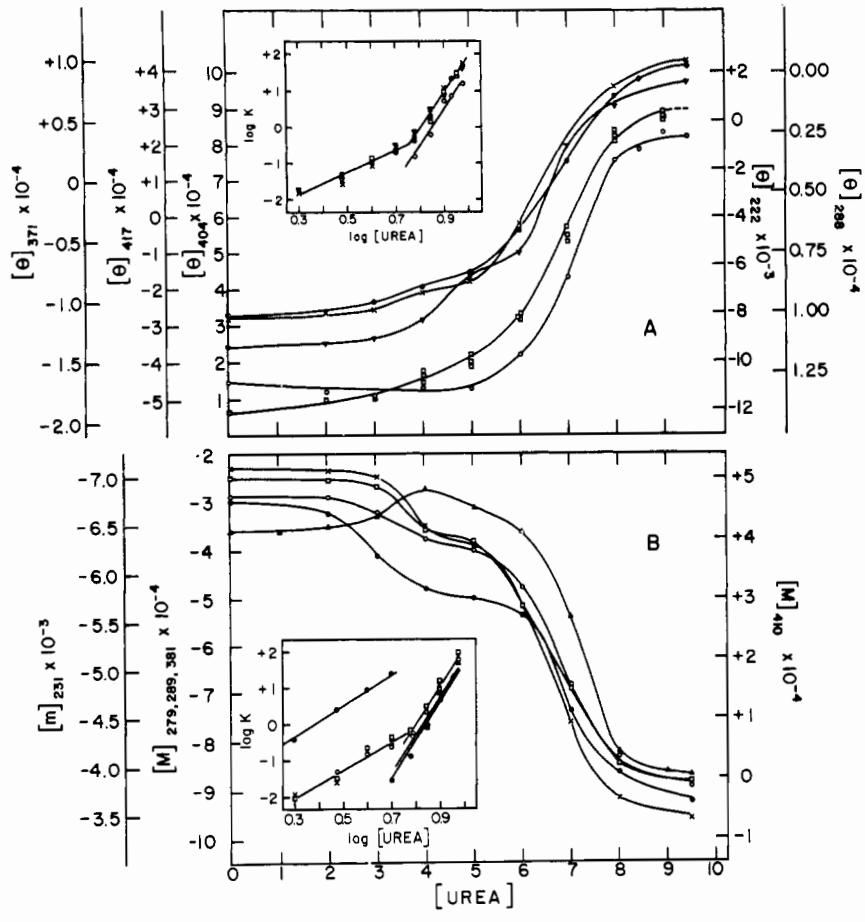


FIGURE 6: Analysis of effect of increasing concentrations of urea on optical rotatory dispersion and circular dichroism curves of horse heart ferricytochrome *c*. Potassium phosphate buffer (0.1 M) and increasing amounts of urea (pH 6.8), temperature, 25°. (A) Ellipticities at: (○) 222, (□) 288, (△) 371, (●) 404, and (×) 417 mμ. (B) Mean residue rotations at 231 mμ, △; and molar rotations at: (×) 279, (□) 289, (○) 381, and (●) 410 mμ. Insets: log — log plots of $K$ with respect to urea concentration, where $K$ is the equilibrium constant between the denatured and the native protein (native protein → denatured protein) and $K = ([\gamma]_\lambda^{\text{native}} - [\gamma]_\lambda^{\text{obsd}})/([\gamma]_\lambda^{\text{obsd}} - [\gamma]_\lambda^{\text{denatured}})$, where $\gamma$ is ellipticity or rotation at wavelength $\lambda$.

including the chemical nature of the iron complex. As shown earlier by Boeri *et al.* (1953) on the bases of spectroscopic investigations of effects of anions in acid solution and recently by Myer *et al.* (1966) from optical rotatory dispersion and spectroscopic investigations under identical conditions, the alteration in the Soret region is an indication of change in the chemical nature of the central coordinated complex, a change of the spin state of heme ion from that of typical high-spin state, containing protonated ligand groups, to one of mixed-spin species, in which the intrinsic ligand groups are held together by two chloride ions. The elimination of the conformation of the polypeptide chain, reflected by changes in the intrinsic absorption region at low ionic strength and its subsequent regeneration upon increase of ionic strength may be attributable to the shielding of the charged group and thus promotion of folding, or the stabilization of the polypeptide conformation by restoration of heme–protein bonds or both (Mirsky and George, 1966; Myer *et al.*, 1966).

The elevation of pH in the range 7–10 alters the dichroic pattern in the intrinsic absorption region to a much smaller extent than in the corresponding acid range; the rotatory strength of the transition exhibiting the 251-mμ peak decreases, and the Soret pattern is transformed into one observed during the thermal transitions of the protein. It may be noted that the contribution from the transition resulting in the 262-mμ peak is lost at alkaline pH but is present in the acid range, though of relatively small magnitude; a reverse situation seems to exist for the band centered at 251 mμ. It may be further pointed out that the rotatory strengths of the 282- and 288-mμ transitions seem to be insensitive to pH variation over approximately the entire pH range (pH 3–11.6) and the only instance

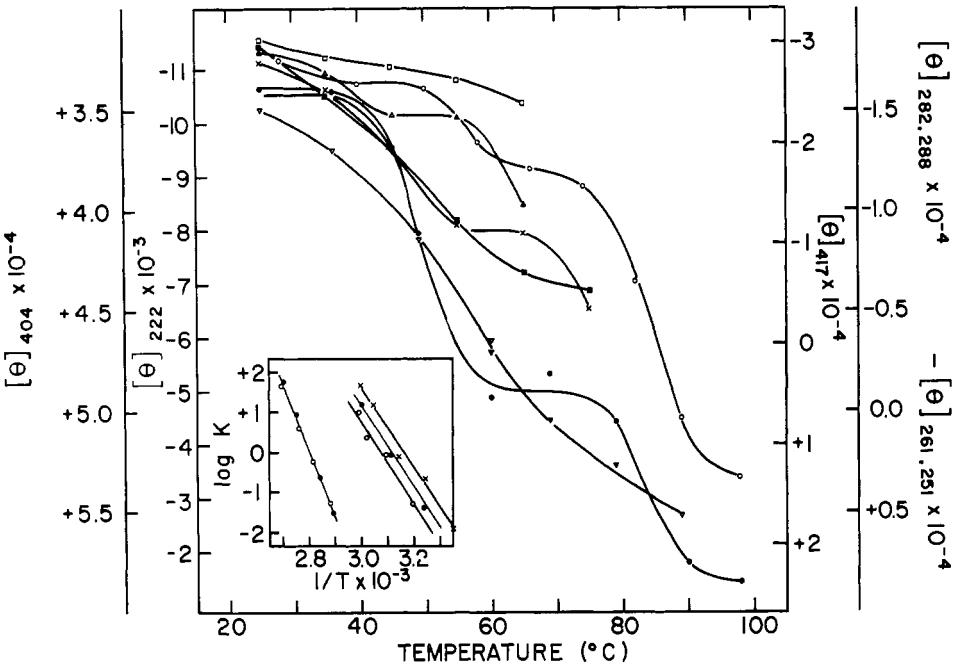


FIGURE 7: Ellipticity at 222, 251, 261, 282, 288, 404, and 417 mμ as a function of temperature, pH 6.8, no buffer. (○) 222, (■) 251, (×) 261, (△) 282, (□) 288, (●) 404, and (▽) 417 mμ. Inset: plots of log $K$ with respect to $1/T$ where $K$ is the equilibrium constant between the denatured and the native protein. $K = ([\theta]_\lambda^{native} - [\theta]_\lambda^{obsd})/([\theta]_\lambda^{obsd} - [\theta]_\lambda^{denatured})$.

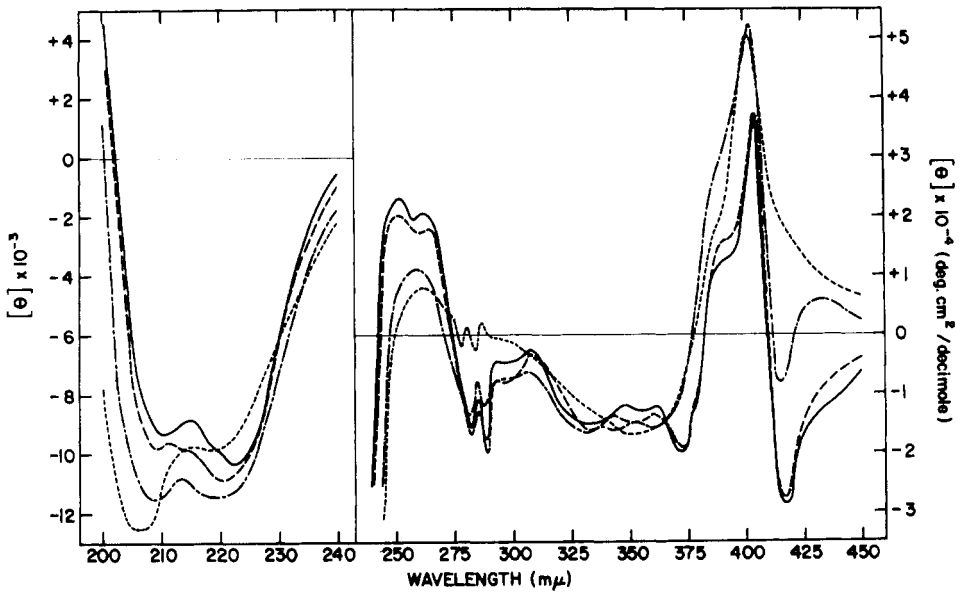


FIGURE 8: Effect of pH on the circular dichroism spectra of horse heart ferricytochrome *c*. Potassium phosphate buffer (0.1 M), pH adjusted with 6 N HCl; temperature, 25°. (———) pH 6.8, (— — —) 5.0, (— · — · —) 3.1, and (– – – – –) 1.6.

when they are affected is when changes are accompanied by alterations in the intrinsic absorption region similar to those of helix–coil transition. Since no unfolding of the polypeptide chain is evident in the pH range 3–11.6, and the alterations of the circular dichroism spectrum over this pH range are similar to those observed during the first step of urea denaturation, the alterations are simply a reflection of changes in the

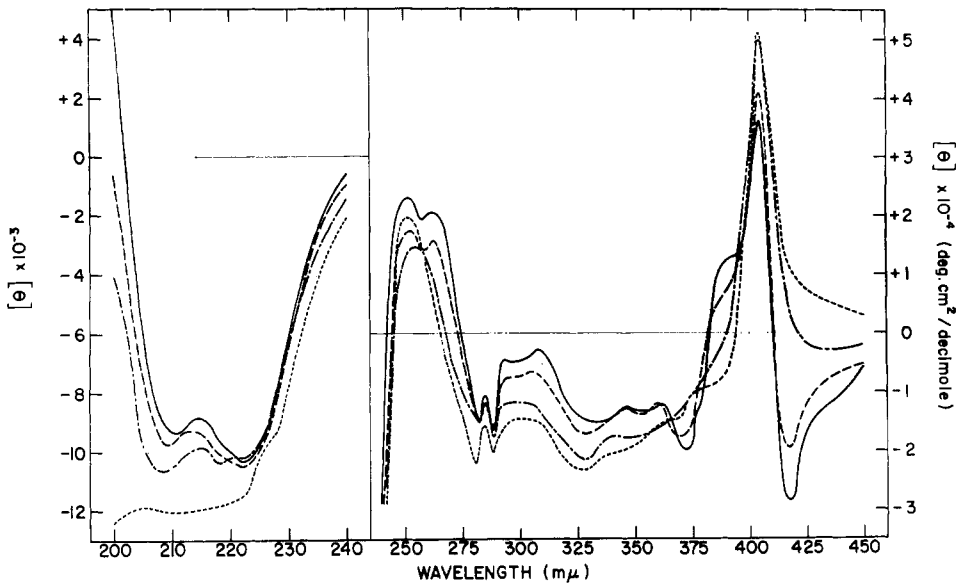


FIGURE 9: Effect of pH on the circular dichroism spectra of horse heart ferricytochrome *c*. Potassium phosphate buffer (0.1 M), pH adjusted with 6 M KOH; temperature, 25°. (———) pH 6.8, (— — —) 8.5, (— — — —) 10.1, and (– – – – –) 11.6.

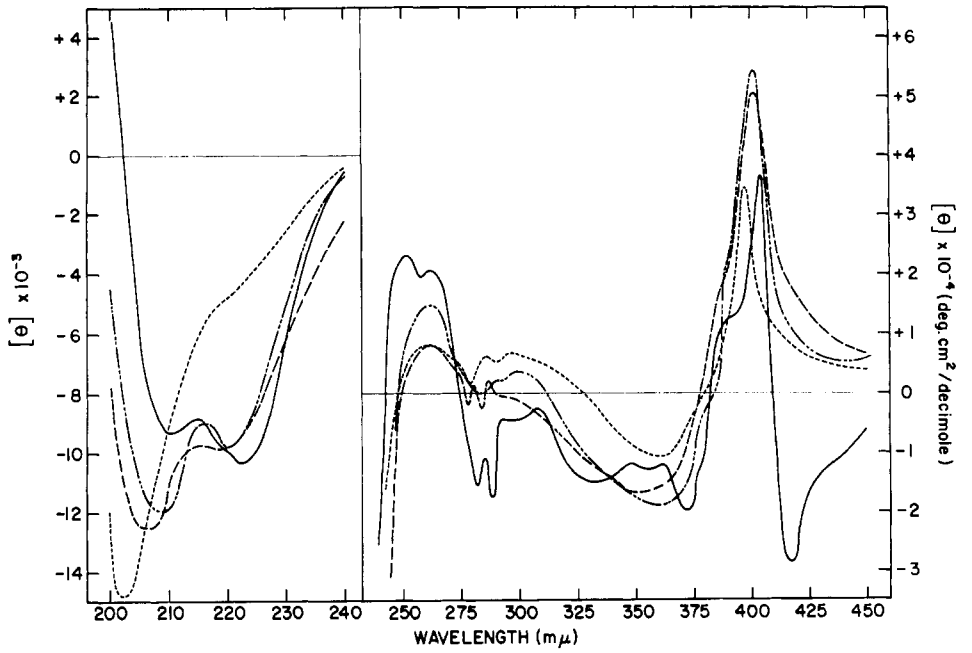


FIGURE 10: Effect of ionic strength on the circular dichroism spectra of horse heart ferricytochrome *c* at acid pH; temperature, 25°. (———) Phosphate-KOH buffer (0.1 M, pH 6.8); (— — —) phosphate buffer and HCl (pH 1.6); (– – – – –) HCl (pH 1.6); (— — — — —) HCl and 0.3 M KCl (pH 1.6).

heme–polypeptide interaction, though different for the acid and the alkali ranges.

Extrinsic ligands to ferricytochrome *c*, such as imidazole, cyanide, and azide, have been shown to replace the intrinsic ligand groups (Horecker and Kornberg, 1946; Horecker and Stannard, 1948; Schejter and George, 1964), and the effects of such replacements on the circular dichroism spectrum of ferricytochrome *c* at pH 6.8 are shown in Figure 11. The dichroic patterns generated in the presence of azide and cyanide are very similar to those observed at alkaline pH values (Figure 9). The shifts in the position of the major

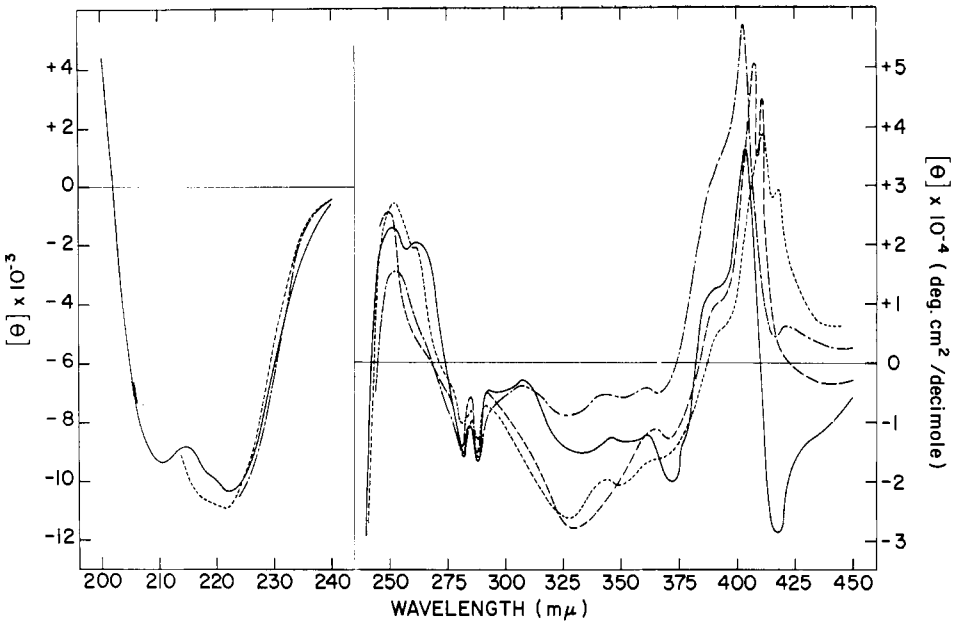


FIGURE 11: Effect of extrinsic ligands on the circular dichroism spectra of horse heart ferricytochrome *c*. Potassium phosphate buffer (0.1 M, pH 6.8); temperature, 25°. (———) No extrinsic ligand; (– – – –) 0.11 M potassium cyanide; (— — —) 0.46 M sodium azide; (— – — – —) 0.54 M imidazole.

Soret dichroic peak (404 mμ) are in line with the known spectral changes induced by these ligands (Horecker and Kornberg, 1946). The addition of these ligands in each instance generates an additional positive dichroic band centered a few millimicrons higher than the major Soret positive peak. The replacement of the intrinsic ligand by an extrinsic ligand group, the disruption of the coordinating bonding between the protein and the heme iron, virtually obliterates the dichroic band at 262 mμ, while those at 251, 282, and 288 mμ remain more or less unchanged. The ellipticity at 222 mμ for the cyanide complex is slightly higher than that of the native protein, which in certain circumstances may be interpreted as a reflection of increase in the helical content of the protein. In the present case, however, the situation is again identical with that encountered during the first step of denaturation of the protein. The increase therefore is the reciprocal effect of alteration of the interactions between the protein and the heme group. The effects of azide and imidazole in the intrinsic absorption region could not be measured due to extensive absorption by these ligands.

The alterations in the wavelength region 300–375 mμ induced by addition of imidazole are significantly different from any of the systems thus far studied (Figures 1–3, 9, and 10). Whether the differences in the 300–375-mμ region are a reflection of differences in the nature of the extrinsic ligand, or of differences in the nature of heme–polypeptide interaction, or of both could not be ascertained with the available data. Further investigations are being conducted on these lines.

It may be pointed out that at concentrations of the ligands required for full conversion of the spectrum, there may occur binding at nonspecific sites in addition to the replacement of the intrinsic ligand group from the heme iron by the added ligand. The conclusions based on the induced alterations should therefore be drawn with caution. The lack of any significant alteration, on the other hand, *e.g.*, of the aromatic dichroic peaks (251, 282, and 288 mμ), may be taken with confidence to indicate that the rotatory strength of the chromophore is not essentially connected with the asymmetric environment of the heme group, an inference contrary to that based on the optical rotatory dispersion studies (Ulmer, 1965).

## Discussion

The optical rotatory dispersion studies of cytochromes thus far undertaken have been predominantly directed toward the elucidation of the oxidation–reduction-linked conformational phenomenon of the protein. Previous optical rotatory dispersion results of cytochrome *c* have shown that the alteration of the oxidation state of heme iron (Urry and Doty, 1965; Ulmer, 1965; Myer and Harbury, 1966; Mirsky and George, 1966), the replacement of the intrinsic ligand by extrinsic ligand in ferricytochrome *c* (Myer and Harbury, 1965; Ulmer, 1966), and the alteration of pH (Myer and Harbury, 1965; Ulmer, 1966; Mirsky and George, 1966) cause major changes in the dissymmetric environment of the heme group, but without any significant alteration of the polypeptide conformation. On the bases of optical rotatory dispersion studies of chemically modified cytochromes *c* (Ulmer, 1966), it has been suggested that the exceptionally large rotatory strengths

of the aromatic chromophores are due to interaction with the prosthetic group, heme. The analysis of the dispersion changes induced by rising temperature, the presence of a two-step thermal transition (Urry, 1965; Myer *et al.*, 1966), and the parallels between the alterations in the intrinsic absorption region and the other parts of the spectrum further indicate the presence of distinct and functional heme–polypeptide or possibly heme–helix interactions. In light of the recent results of the studies of crystal structure of ferricytochrome *c* (Dickerson *et al.*, 1967) and especially the richness of dichroic spectra (Y. P. Myer, unpublished data), the investigations outlined here were undertaken to see whether the conclusions based on optical rotatory dispersion studies, in which some of the Cotton effects of fairly large rotatory strengths went completely undetected, *e.g.*, 262- and 251-m$\mu$ bands, could be confirmed.

The denaturation of horse heart ferricytochrome *c*, whether brought about by elevation of temperature or by addition of increasing concentrations of urea, whether determined by alteration of rotations or of ellipticities at the extrema in the intrinsic absorption region (reflecting the alteration of the polypeptide conformation), in the Soret absorption region (predominantly ligand–porphyrin transitions), or in the aromatic absorption region (possibly containing both the aromatic and the heme transitions), occurs in two distinct steps (Figures 6 and 7). The identity of slopes of log $K$ *vs.* $1/T$, where $K$ is the equilibrium constant between the denatured and the native protein, both from the data in the intrinsic absorption region (222 m$\mu$) and from the visible and the near-ultraviolet regions, representing alteration of the dissymmetric environment of the chromophore (*cf.* inset of Figure 7), and similar results for the data for urea denaturation, at least for the second step of the process (*cf.* inset of Figure 6), and furthermore, the persistence of the identity of slopes irrespective of the wavelength for each of the steps, indicate that the rotatory strengths of almost all the transitions, whether arising from the prosthetic group or from the aromatic amino acid side chains or the peptide bonds, are linked with each other, and the two steps of the denaturation thus reflect the preferential alteration of the interplay between the protein and the prosthetic group, heme, and of the unfolding of the polypeptide chain. The small increase in the rotatory strength of the 222-m$\mu$ dichroic band and the concomitant decrease in the rotatory strengths of transitions in other parts of the spectrum, especially the 450-, 417-, and the 372-m$\mu$ dichroic bands, during the first step of urea denaturation are consistent with the expected behavior of transitions deriving their rotatory strengths from electric–electric dipole interactions (the reciprocal relationship being a corollary of the sum rule based on the quantum-mechanical treatment of the coupled oscillator) (Condon, 1937; Kuhn, 1930; Urry, 1965). The coupled transitions in this case seem to be those of the protein in $\alpha$-helical conformation and the prosthetic group, heme. The rotatory strength of the 222-m$\mu$ ellipticity band during the first step of thermal transition, however, decreases, whereas the trend of changes in the visible part of the spectrum is similar for both the processes. The decrease of ellipticity at this wavelength could very easily be due to the opposing contributions from the unfolding of a segment of the polypeptide chain (Urry, 1965). Since the heating of a protein enhances the unfolding in general because of the large positive entropy, 150 eu for the first step and about 220 eu for the second step, and whereas urea presumably causes unfolding by competition with various stabilizing bonds, *viz.*, peptide–hydrogen bonds and the side-chain bonds, etc., there is a distinct possibility that a certain section of the polypeptide chain may undergo unfolding from rising temperatures and not from an initial increase of the urea concentration. The first step of urea unfolding is therefore interpreted as predominantly the displacement of the polypeptide chain from the close proximity of the heme group resulting in obliteration of heme–helix interaction, and the first step of thermal denaturation as the composite of unfolding of a section of the polypeptide chain and the uncoupling of the transitions (Urry, 1965).

For reasons given above, which indicate that the initial thermal denaturation step is the composite of the unfolding of the polypeptide chain and the uncoupling of the heme–helix interaction, and from the comparison of the magnitudes of the changes in the intrinsic absorption region to those in the Soret and other parts of the spectrum (Figure 7), it is evident that the second step of thermal denaturation is predominantly the unfolding of the polypeptide chain, which results in further simplification of the Soret dichroic pattern. At the highest temperature thus far investigated (98°), the large dichroic absorption at 222 m$\mu$ is unaccountable on the basis of contributions from the model random polypeptides (Holzwarth and Doty, 1965), possibly reflecting only the partial unfolding of the polypeptide chain at this temperature. This is also indicated by the observation of a well-defined rotatory minimum at about 231 m$\mu$, with no evidence of the development of the 210-m$\mu$ levorotatory trough (Myer *et al.*, 1966), a characteristic of simple random polypeptide systems (Simmons *et al.*, 1961). However, in 9.5 M urea there seems to be almost complete unfolding of the polypeptide chain, reflected by the small magnitude of ellipticity at 222 m$\mu$ (Figure 1), comparable to that of random polypeptides;[2] in addition the ellipticity at 404 m$\mu$ is approximately twice the magnitude observed at elevated temperatures. Therefore it appears that the second step of the denaturation process induced by urea is significantly different from that observed by elevation of temperatures.

The hyperchromism and the hypsochromic shifts of the Soret band and the concomitant hypochromism of the 528-m$\mu$ band upon addition of urea (Figures 4 and 5) are typical of the alteration of the spin state of heme iron from a typical low-spin state to mixed-spin state (Boeri *et al.*, 1953). A change in the coordinating groups of the heme iron from position 5 or 6 or from both is therefore indicated. A similar conclusion has

also been reached by Stellwagen (1967) from the studies of solvent perturbation of ferricytochrome *c*. The effect of elevation of temperatures on the absorption spectrum, on the other hand, seems to be devoid of any indication of either the alteration of the intrinsic ligand or the spin state of the heme iron. Since the midpoint of the spectroscopic titration by urea is located at about the same urea concentration as observed for the second step of denaturation (*cf.* inset of Figure 4; Figure 6), it seems that there exists essentially complete overlap between the second step of urea denaturation and the transition involving the alteration of the chemical nature of the central coordinated complex. The differences between the second steps of thermal and urea denaturation may, therefore, be attributable to the additional degree of freedom imposed by the disruption of the bonding between protein and heme iron, and thereby the extensive unfolding of the polypeptide chain and the simplification of the Soret dichroic pattern. The second step of urea denaturation is therefore a composite of two major effects, namely, the replacement of the intrinsic ligand by extrinsic ligands and the extensive unfolding of the polypeptide chain.

If the above interpretation of differences between thermal and urea denaturation is accepted, then it seems that the coordination of the intrinsic ligand groups results in stabilization of the conformation of a certain section or sections of the polypeptide chain. The sequence involved may simply be more conformationally stable to thermal denaturation, as in the case of $\beta$ structure. There seems to be a distinct possibility also that the conformation of the polypeptide in close proximity to the coordinating amino acid is rather stable to unfolding by elevation of temperatures because of relatively strong coupling of the transitions, or by hydrogen bonding with the propionate side chains of the heme group, or by the rigidity imposed by the two thioether bridges linking the heme group to the polypeptide chain, or a combination of events. In addition, the question arises whether the disruption of the coordinating linkage at position 5, the position occupied by imidazole of histidine residue 18 (Margoliash and Schejter, 1966) or at position 6, possibly occupied by methionine residue 80 (Matsubara *et al.*, 1965; Schejter and George, 1965; Harbury *et al.*, 1965; Fanger *et al.*, 1967) or of both results in the destabilization of the polypeptide conformation. X-Ray diffraction studies and further circular dichroism investigations may assist in the resolution of this aspect of the structure–conformation relationship of the protein.

The exposure of horse heart ferricytochrome *c* to various pH values in the range 3–10 (Figures 8 and 9) or to the heme-binding ligands such as azide, cyanide, or imidazole (Figure 11) results in simplification of the Soret dichroic pattern similar to that observed during the initial step of urea denaturation. The changes in the intrinsic absorption region also follow the same pattern. By analogy with the earlier conclusions, it seems that in each of the instances, there occurs mainly the weakening of the interactions between the protein and the prosthetic group. The change in the trend of the alterations of the circular dichroism spectrum below pH 3 and above pH 10 presumably reflects the formation of type II and type IV compounds according to the classification of Theorell and Akesson (1941) based on the changes in the absorption spectrum. The effect of ionic strength on the circular dichroism spectrum at pH 1.6 (Figure 10) seems to reflect alterations basically similar to those observed during rotatory dispersion studies under identical conditions (Myer *et al.*, 1966; Mirsky and George, 1966) and therefore supports earlier conclusions.

*The Circular Dichroism Spectrum in the Region 300–245 mμ.* Several lines of evidence lead to the conclusion that the contributions to the 288-, 282-, and 251-mμ ellipticity bands are generated by the aromatic side chains, and that the 262-mμ band arises from the heme group. The single tryptophan side chain seems to be the likely candidate for the presence of the 288- and 282-mμ bands because of the identity of the wavelengths with those observed for simple free amino acid (Y. P. Myer, unpublished data). The presence of a positive band at about 245 mμ in the circular dichroism spectrum of poly-L-tyrosine films of the free acid and of a positive band of comparable size both in the random and the helical amino acid polymer (Beychok and Fasman, 1964) suggest that the tyrosine side chains are the possible chromophores generating the 251-mμ band. Further support to the above is provided by the circular dichroism spectra of simple heme systems lacking these chromophores. The circular dichroism spectra of heme–octapeptide (a simple heme–peptide segment from horse heart cytochrome *c* containing eight amino acids residues from 11 to 18) and of heme–undecapeptide (containing residues from 10 to 20) in the aromatic absorption region do not show any evidence of dichroic bands at wavelengths 288, 282, and 251 mμ (Zand and Vinogradov, 1967; Urry, 1967; Y. P. Myer, unpublished data). Since the model systems contain the prosthetic group, the histidine side chain, and the two thioether bridges, but lack the amino acids with aromatic side chains, the origins of the 288-, 282-, and 251-mμ bands are thus suggested. The circular dichroism spectra of the two model systems, however, show a well-defined positive dichroic peak centered at about 262 mμ, thus suggesting the heme origin of the 262-mμ band. The presence of a dichroic band at the identical position in the dichroic spectrum of hemoglobin and its absence in the spectrum of the native globin (Beychok *et al.*, 1967) further support the dominant role of heme in governing the 262-mμ band.

Since the rotatory strength of the transition of the chromophore and the sign of the dichroic band serve as a measure of the interaction of the chromophore with its asymmetric environment and the concomitant asymmetry induced in the electron distribution within the chromophore, a comparison of parameters with those of the model systems, aromatic amino acids or their homopolymers, would be useful for the understanding of the spatial implications of these chromo-

phores. The rotatory strength as well as the sign of the 251-mμ band is comparable to a corresponding band in poly-L-tyrosine (Beychok and Fasman, 1964), if equal contribution is assigned to each of the four tyrosine side chains of the protein. In the case of free amino acid, however, there seems to be no ellipticity band corresponding to this wavelength, except for a relatively small band at about 245 mμ in ionized L-tyrosine. The tryptophan content of the protein is about tenfold too small to account for the ellipticity at 288 and 282 mμ (Y. P. Myer, unpublished data). In addition, the sign of the dichroic band is negative, whereas that exhibited by a free amino acid is positive. The anomalous rotatory strengths and the reversed sign of the dichroic bands, therefore, indicate that cytochrome *c* endows the chromophores of the aromatic amino acids with an unusual dissymmetric environment.

The relatively low sensitivity of the 282- and 288-mμ ellipticity bands to alteration of interplay between the protein and the prosthetic group when not accompanied by changes in the conformation of the polypeptide chain, such as (a) the alteration of pH between 3 and 11.6 (*cf.* Figures 8 and 9); (b) the disruption of the protein–heme iron bonds by extrinsic ligands (*cf.* Figure 11); and (c) the elevation of temperatures compatible with the first stem of thermal denaturation (*cf.* Figure 3), and the obliteration or decrease of ellipticity at these wavelengths following alteration accompanying the unfolding of the polypeptide chain (in 9.5 M urea; at pH 1.5, Figures 9 and 10) clearly indicate that the rotatory strengths of these transitions are not significantly dependent on the interplay between the heme and the polypeptide chain, but are governed by the folding of the protein moiety, perhaps through stacking of the rings (Fasman *et al.*, 1964) or through appropriate orientation or the dissymmetry arising from the local distribution of the charged groups.

The ellipticity of the 251-mμ band, on the other hand, shows remarkable sensitivity to not only the interplay between the protein and the prosthetic group (*cf.* Figures 8–11), but also to the unfolding of the polypeptide chain (Figures 3 and 10). The changes in the 262-mμ ellipticity band, in almost all instances, seem to follow the changes in the Soret region, and thus the analysis based on the alteration at this wavelength would yield conclusions similar to those already stated. However, the exceptional sensitivity of the 262-mμ band to alteration of the heme environment (*cf.* Figures 3 and 9), to the specific nature of the coordinating ligand (*cf.* Figures 10 and 11), to the spin state of the heme iron (Figure 10) and to its oxidation state (Y. P. Myer, unpublished data), and the relatively favorable dichroic:optical absorption ratio, in comparison to the Soret absorption band, should provide a useful tool for conformation investigations of hemoproteins.

References


Beychok, S., and Fasman, G. D. (1964), *Biochemistry 3*, 1675.

Beychok, S., Tyuma, I., Benesch, R. E., and Benesch, R. (1967), *J. Biol. Chem. 242*, 2460.

Boeri, E., Ehrenberg, A., Paul, K. G., and Theorell, H. (1953), *Biochim. Biophys. Acta 12*, 273.

Condon, E. U. (1937), *Rev. Mod. Phys. 9*, 432.

Dickerson, R. E., Kopka, M. L., Weinzierl, J. E., Varnum, J. C., Eisenberg, D., and Margoliash, E. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept, Abstr C1.

Fanger, N. W., Hettinger, T. P., and Harbury, H. A. (1967), *Biochemistry 6*, 713.

Fasman, G. D., Bodenheimer, E., and Lindblow, C. (1964), *Biochemistry 3*, 1665.

Harbury, H. A., Cronin, J. R., Fanger, M. W., Hettinger, T. P., Murphy, A. J., Myer, Y. P., and Vinogradov, S. N. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1658.

Holzwarth, G., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 218.

Horecker, B. L., and Kornberg, A. (1946), *J. Biol. Chem. 165*, 11.

Horecker, B. L., and Stannard, J. M. (1948), *J. Biol. Chem. 172*, 589.

Kuhn, W. (1930), *Trans. Faraday Soc. 26*, 293.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

Matsubara, H., Ando, K., Okunuki, K. (1965), *Proc. Japan Acad. 41*, 408.

Mirsky, R., and George, P. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 222.

Myer, Y. P., and Harbury, H. A. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1391.

Myer, Y. P., and Harbury, H. A. (1966), *J. Biol. Chem. 241*, 4299.

Myer, Y. P., Murphy, A. J., and Harbury, H. A. (1966), *J. Biol. Chem. 241*, 5370.

Schejter, A., and George, P. (1964), *Biochemistry 3*, 1045.

Schejter, A., and George, P. (1965), *Nature 206*, 1150.

Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Stellwagen, E. (1967), *J. Biol. Chem. 242*, 602.

Theorell, H., and Akesson, A. (1941), *J. Am. Chem. Soc. 63*, 1812.

Ulmer, D. D. (1965), *Biochemistry 4*, 902.

Ulmer, D. D. (1966), *Biochemistry 5*, 1886.

Urry, D. W. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 640.

Urry, D. W. (1967), *J. Am. Chem. Soc. 89*, 4190.

Urry, D. W., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 2756.

Zand, R., and Vinogradov, S. N. (1967), *Biochem. Biophys. Res. Commun. 26*, 121.