Optical Rotatory Dispersion of Oxidized and Reduced Cytochrome *c**

David D. Ulmer†

ABSTRACT: Oxidation-reduction of cytochrome *c* is accompanied by marked changes in optical rotatory dispersion. The principal deflection of the *extrinsic* Cotton effect arising from the Soret band of the oxidized protein is positive; in the reduced cytochrome it is negative. The two proteins also exhibit pronounced differences in the extrinsic Cotton effects generated by the other absorption bands of the iron-porphyrin moiety as well as in the anomalous dispersion which arises from the side chain chromophores and conformational features of the protein. In this regard, the rotatory dispersion of the oxidized cytochrome reveals two peaks in the area of absorption of the aromatic amino acids, while the reduced protein exhibits only one. Further, the *intrinsic* Cotton effect of reduced cytochrome *c* is of slightly greater magnitude than that of the oxidized protein.

These observations indicate that oxidation-reduction is accompanied both by alterations in protein conformation and by a change in orientation of the heme with respect to the protein axis. An interaction between the iron-porphyrin group and the aromatic amino acid residues is suggested by the demonstration of simultaneous alterations in their characteristic rotatory parameters as a consequence of changes in oxidation state.

A number of observations suggest that oxidized and reduced cytochrome *c* differ in structure. Thus in some species oxidized cytochrome is more likely to undergo spontaneous changes in physical state than is the reduced protein (Hagihara *et al.*, 1958) and is less resistant to proteolytic digestion (Nozaki *et al.*, 1958). Electrometric titration of the oxidized and reduced forms of the protein reveal an oxidation-linked ionization near *p*H 8 (Rodkey and Ball, 1950). Moreover, while oxidized cytochrome *c* binds to xanthine oxidase, reduced cytochrome *c* does not (Fridovich, 1962). The physical basis for these differences in behavior has not yet been delineated.

Spectropolarimetry would appear to provide an especially suitable method for the extension of these observations. During the past few years optical rotatory dispersion has been shown to serve both as a sensitive index of alterations in protein conformation (Blout, 1960; Urnes and Doty, 1961; Schellman and Schellman, 1964) and of changes in the symmetry of *localized* areas of protein structure (Ulmer and Vallee, 1963, 1965). In heme proteins, for example, the chromophoric iron-porphyrin moiety is sterically oriented by the protein ligands of the metal atom and the porphyrin side chains and generates pronounced Cotton effects (Urnes *et al.*, 1961; Beychok and Blout, 1961; Eichhorn, 1961; Ulmer and Vallee, 1963; Yang and Samejima, 1963). Any subtle alterations in the asymmetry of this structure, consequent to a change in the oxidation state of the iron, might be manifested by changes in rotatory dispersion (Ulmer and Vallee, 1965).

The optical rotatory properties of reduced and oxidized cytochrome *c* are reported in the present paper. In addition, the recent isolation and characterization of soluble, enzymatically active phospholipid–cytochrome *c* complexes (Widmer and Crane, 1958; Das and Crane, 1964) has provided the impetus to examine the rotatory dispersion of this protein under conditions which may, in certain respects, approximate the subcellular environment.

Materials and Methods

Horse heart cytochrome *c* (Type III) was obtained from Sigma Chemical Co. and isooctane (2,2,4-trimethylpentane) from Eastman Chemical Co. Mixed phospholipids were a gift of Prof. A. Neuberger.

Oxidized cytochrome *c* was prepared by exposing samples of the protein to 0.001 M ferricyanide for 2 hours, followed by extensive dialysis against several changes of 0.1 M sodium phosphate buffer, *p*H 6.8. The protein was reduced either by treatment with dithionite and subsequent dialysis to remove the excess reducing agent, or catalytically using platinum black and hydrogen gas. The spectral properties of the oxidized and reduced proteins conformed closely to those described by Margoliash and Frohwirt (1959).

Phospholipid–cytochrome *c* complexes were prepared as described by Das and Crane (1964). Stoichiometric complexes with a ratio of phosphorus to cyto-

* From the Biophysics Research Laboratory, Harvard Medical School, and the Department of Medicine, Peter Bent Brigham Hospital, Boston, Mass. *Received March 17, 1965.* This work was supported by grants-in-aid (GM-11639-02 and HE-07297) from the National Institutes of Health of the U.S. Department of Health, Education and Welfare.

† Research Career Development Awardee of the National Institutes of Health of the U.S. Department of Health, Education and Welfare.

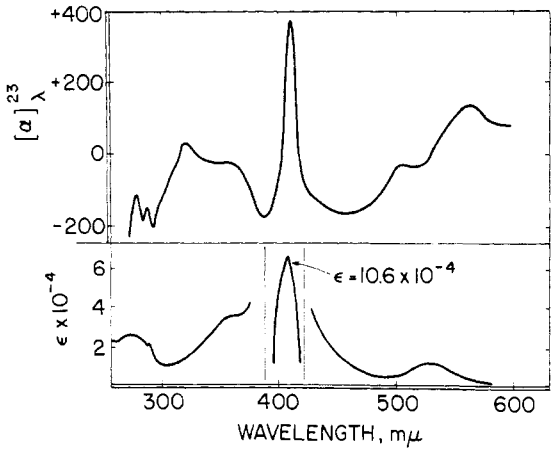

FIGURE 1: Optical rotatory dispersion and absorption spectrum of oxidized cytochrome *c*. Specific rotation, $[\alpha]^{23}$, and molar absorptivity, ϵ, are plotted against wavelength. The Soret band at 407 mμ generates a marked *positive* extrinsic Cotton effect. Extrinsic Cotton effects also arise from the heme absorption bands at 528 and 360 mμ. Peaks at 278 and 287 mμ are associated with the chromophores of the aromatic amino acids. Conditions: 2–3 mg/ml protein in 0.1 M sodium phosphate, *p*H 6.8. In this preparation of oxidized cytochrome, $\epsilon = 1.07 \times 10^{-4}$ at 528 mμ and 0.775×10^{-4} at 550.25 mμ.

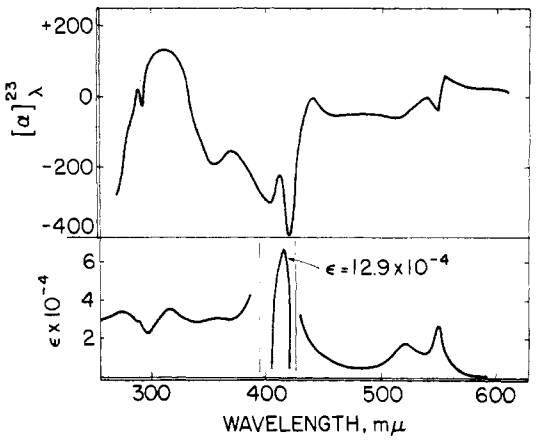

FIGURE 2: Optical rotatory dispersion and absorption spectrum of reduced cytochrome *c*. Specific rotation, $[\alpha]^{23}_{\lambda}$, and molar absorptivity, ϵ, are plotted against wavelength. The principal deflection of the extrinsic Cotton effect arising from the Soret band, at 413 mμ, is *negative*. Pronounced Cotton effects, which differ both in configuration and in wavelength from those in the oxidized cytochrome, are generated by the heme absorption bands at 550.25 and 315 mμ. In contrast to the multiple Cotton effect associated with the chromophores of the aromatic amino acids of the oxidized protein, only one peak, that at 287 mμ, is present in reduced cytochrome *c*. Conditions: 2–3 mg/ml protein in 0.1 M sodium phosphate, *p*H 6.8. The cytochrome was reduced catalytically by hydrogen in the presence of platinum black. Identical results are obtained when the protein is reduced with dithionite. In this preparation of reduced cytochrome, $\epsilon = 0.89 \times 10^{-4}$ at 528 mμ and 2.44×10^{-4} at 550.25 mμ.

chrome of 24:1 to 32:1 were obtained. Phosphorus was determined according to a modification of the method of Ames and Dubin (1960). Cytochrome *c* concentrations were determined spectrophotometrically based on the absorbance at 526 mμ, the isosbestic point for the oxidized and reduced proteins, and at the Soret bands (Margoliash and Frohwirt, 1959).

Optical rotatory dispersion was measured in the Cary Model 60 recording spectropolarimeter over the spectral range 190–620 mμ, at a temperature of 23°. Cells with fused quartz end-plates and 0.1–2 mm path length were employed for most determinations; protein concentrations varied from 0.3 to 3 mg/ml. When more dilute solutions of phospholipid–cytochrome *c* were analyzed, cells of up to 5 cm in path length were employed. The slit width of the instrument was programed to yield maximal and constant light intensities at all wavelengths. In areas of high absorbance, absolute values for specific rotation were confirmed at two or more protein concentrations or path lengths, eliminating the possibility of spurious Cotton effects (Urnes and Doty, 1961). Specific rotations were calculated on the basis of protein concentration.

Results

The optical rotatory dispersion and absorption spectra of oxidized cytochrome *c* in 0.1 M sodium phosphate buffer, *p*H 6.8, are shown in Figure 1. The rotatory dispersion curve is rich in experimental detail, as is characteristic for heme proteins (Beychok and Blout, 1961; Ulmer and Vallee, 1963; Yang and Samejima, 1963). In addition to the marked positive *extrinsic* Cotton effect[1] associated with the Soret band at 407 mμ, anomalous dispersion is generated by the broad porphyrin absorption band at 528 mμ and by the shoulder at 360 mμ. Two sharply defined peaks, at 287 and 278 mμ, respectively, arise from the absorption bands of the aromatic amino acids and suggest that cytochrome *c* provides an unusually pronounced asymmetric environment for these chromophores. The oxidation state of the iron atom in the heme group has a marked influence on all these Cotton effects.

The optical rotatory dispersion and absorption spectra of reduced (platinum black/hydrogen gas) cytochrome *c* are shown in Figure 2. In marked contrast to

[1] The terminology is in accord with the suggestion of Blout (1964), where *intrinsic* Cotton effects are those which appear to arise from the primary and secondary structure of proteins, e.g., through conformational orientation of the peptide bonds, while Cotton effects which are generated by prosthetic groups, bound cofactors, or metal atoms, for example, are designated as *extrinsic*.

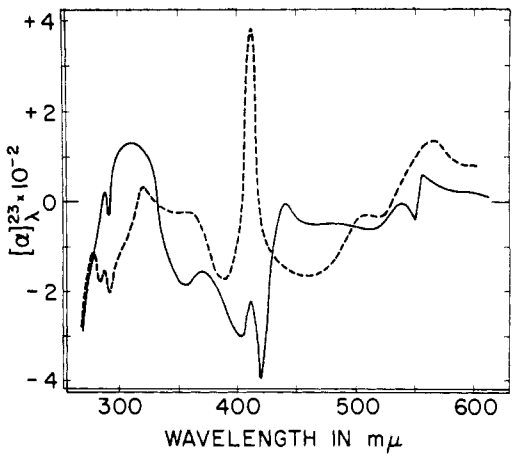

FIGURE 3: Comparison of the extrinsic Cotton effects of oxidized cytochrome *c*, (- - - -), with those of the reduced protein, (———). Specific rotation, $[\alpha]_\lambda^{23}$, is plotted against wavelength. During oxidation-reduction both the configuration of these effects and the wavelengths of their inflection points changed in accord with corresponding shifts in the absorption maxima. The data are taken from those shown in Figures 1 and 2.

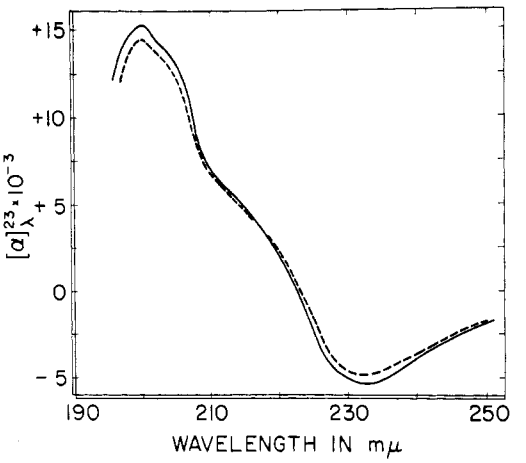

FIGURE 4: Intrinsic Cotton effects of oxidized, (- - - -), and reduced, (———), cytochrome *c*. The specific rotation, $[\alpha]_\lambda^{23}$, is plotted against wavelength. In multiple experiments the magnitude of the intrinsic Cotton effect of reduced cytochrome *c* is slightly but consistently greater than in the oxidized cytochrome. Conditions: 0.3–0.5 mg/ml protein in 0.1 M sodium phosphate, *p*H 6.8.

the oxidized protein, the principal deflection of the Soret band Cotton effect of reduced cytochrome *c* is *negative*, producing a deep trough at 420 mμ. The *initial* deflection of this effect appears to be a low peak near 440 mμ; a second peak, at 410 mμ, is similar in breadth and position to that observed in the oxidized protein, though it is of lesser magnitude. In addition to these alterations at the Soret band, the characteristic α-absorption band of reduced cytochrome *c*, at 550.25 mμ, generates a remarkably sharp positive Cotton effect as noted previously by Eichhorn and Cairns (1958). A positive Cotton effect with a peak near 320 mμ, just discernible in the oxidized protein (Figure 1), is significantly increased in magnitude upon reduction of cytochrome *c;* thus it appears to arise from the δ-absorption band at 315 mμ. While oxidized cytochrome *c* exhibits *two* distinct maxima in the region of 270–300 mμ, in which the aromatic amino acids absorb radiation, only one of these, the peak at 287 mμ, is preserved in reduced cytochrome *c*. Thus the asymmetric chromophore which gives rise to the peak at 278 mμ appears to be linked to the oxidation state of the iron atom. To facilitate direct comparison of the extrinsic Cotton effects of the oxidized and reduced proteins their optical rotatory dispersion curves are juxtaposed in Figure 3.

The *intrinsic* Cotton effects of oxidized and reduced cytochrome *c* are very similar (Figure 4). However, in the reduced protein the trough at 233 mμ is slightly deeper and the peak at 200 mμ somewhat higher than in the oxidized cytochrome. This difference in rotation for the two proteins averages about 4% both at the peak and at the trough of the intrinsic Cotton effect. Although the insensitivity of the method precludes a valid statistical evaluation, the trend of the observations has been sustained in multiple experiments and suggests that small changes in protein conformation may occur consequent to change in the oxidation state of the iron atom.

Phospholipid–cytochrome *c* complexes were prepared from aqueous protein solutions, in the absence of any buffer salts, and without prior exposure to either oxidizing or reducing agents. The initial aqueous preparation, therefore, is largely (about 90%) in the oxidized form as supplied commercially. In Figure 5 the rotatory dispersion of such a preparation of cytochrome *c* in water is compared with that of a phospholipid–cytochrome *c* complex, extracted into isooctane. Notably, the rotatory properties of the lipid-protein complex in organic solvent are virtually identical to those of the aqueous protein solution and are closely similar to those of the oxidized protein in 0.1 M sodium phosphate buffer, shown in Figure 1. Moreover, there is no discernible change in the magnitude of the trough of the intrinsic Cotton effect of cytochrome *c*, at 233 mμ, upon formation of the phospholipid-protein complex. Variations in protein structure, as evidenced by means of spectropolarimetry, could not be detected in preparations having ratios of phosphorus to cytochrome *c* ranging from 24:1 to 32:1.

Discussion

It is generally accepted that the biological functions of proteins will ultimately be accounted for in terms of their composition and structure. Delineation of these properties presently constitutes a most active and productive area of investigation. Cytochrome *c*,

the best-characterized member of the functional unit known as the terminal oxidation chain, has long served as an important example in studies relating biological structure and function.

Major emphasis has been given lately to investigation of changes in function which result from modification of primary, secondary, tertiary, and quaternary protein structure. Though structural alterations have long been suspected to accompany normal variations in biologic state or activity, it has been difficult to demonstrate such subtle changes conclusively. These considerations are directly pertinent to the spectropolarimetric properties of reduced and oxidized cytochrome *c*.

The complex anomalous rotatory dispersion of cytochrome *c* reflects contributions to rotation both of the iron-porphyrin groups, primarily at wavelengths longer than 300 mμ, and of the protein, at shorter wavelengths. Contributions from *both* sources are influenced by the oxidation state of the iron atom.

The principal deflection of the Cotton effect arising from the Soret band, positive in the oxidized protein, becomes negative when cytochrome *c* is reduced. Alterations of lesser magnitude are associated with the other absorption bands generated by the iron-porphyrin moiety. Hence, oxidation-reduction of cytochrome *c* appears to change the orientation of the heme group with respect to the protein chains. In addition to the altered anomalous dispersion arising from the metal-porphyrin absorption bands, however, reduction of cytochrome *c* induces a small increase in amplitude of the *intrinsic* Cotton effect thought to reflect the degree of protein helicity (Blout *et al.*, 1962; Blout, 1964). Moreover, a peak at 278 mμ, observed in the oxidized protein, is absent in reduced cytochrome *c*, indicating a variation in the asymmetry of the aromatic amino acid chromophores. Taken together these alterations are consistent with a change in protein conformation consequent to oxidation-reduction of the iron atom in the heme group. Moreover, an interaction between the iron-porphyrin moiety and the aromatic amino acid side chains is suggested by the demonstration of simultaneous alterations in their characteristic rotatory parameters accompanying changes in oxidation state.

While the present data alone do not establish different protein structures in oxidized and reduced cytochrome with certainty, this appears to be a most likely interpretation and, currently, is the subject of further investigation (D. D. Ulmer, work in progress). Such a view is supported by the recent demonstration that removal of the heme moiety of myoglobin appears to result in a substantial decrease in helicity of that protein (Harrison and Blout, 1965; Breslow *et al.*, 1965). While this modification would clearly be much more drastic than the oxidation-reduction here studied, it would be anticipated that a change in orientation of the heme group might cause an analogous, albeit less pronounced, structural alteration as, indeed, appears to occur in cytochrome.

This interpretation is also consistent with the previous and independent observations of a differential sensitivity to proteolytic digestion (Nozaki *et al.*, 1958) and of

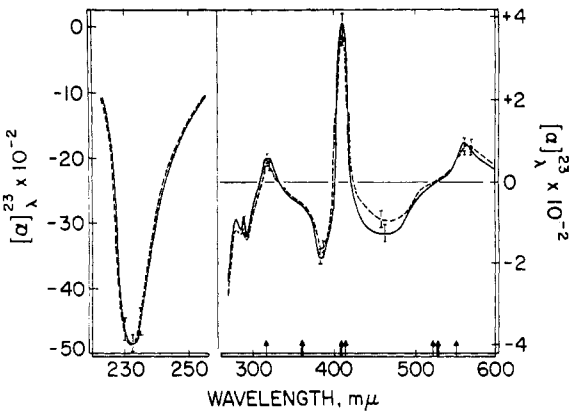

FIGURE 5: Optical rotatory dispersion of cytochrome *c* and its phospholipid complex. Specific rotation, $[\alpha]^{2}_{\lambda}$, is plotted against wavelength. Heavy arrows along the abscissa indicate absorption maxima of the oxidized cytochrome and light arrows indicate those of reduced cytochrome. Within the limits of experimental error, no differences are detected between the rotatory dispersion of cytochrome *c* in water, (———), and the cytochrome *c*–phospholipid complex in isooctane, (– – – –). Protein concentrations are 0.12–0.45 mg/ml.

binding to xanthine oxidase (Fridovich, 1962) for the two oxidation states of cytochrome *c*. Presumably, only small alterations of structure are needed to account for such differences in behavior of these two states of the protein if it is recalled that various enzymes have been shown to be protected against either proteolysis or chemical modifications by the presence of their coenzymes or substrates (Yonetani and Theorell, 1962; Sund and Theorell, 1963; Li and Vallee, 1964). Significant changes might be confined to a localized site of the protein, perhaps directly contiguous to the heme group and accounting for the observations.

In this regard, it has been suggested that a conformational change in cytochrome *c*, induced by oxidation-reduction, might be a structural feature which serves as a functionally essential link of oxidative phosphorylation. Such a change could be mediated by a switchover to a different donor group for the iron atom during the oxidation-reduction cycle (George and Glauser, 1959; George, 1961).

It has also been thought that the peptide chain in cytochrome *c* may be fairly closely packed and that the heme disk may parallel the axis of the α-helix (Ehrenberg and Theorell, 1955; Blauer and Ehrenberg, 1963). Accepting such hypotheses, it could be visualized readily that a change in bonding of the iron atom during oxidation-reduction reorients the plane of the heme atom, with respect to the helical axis, and this circumstance could then account for the change in sign of the Cotton effect at the Soret band of cytochrome *c*. Such an inversion in the sign of the Cotton effect might also be related to alterations in protein helicity, to changes in the geometry of coordination by the metal atom, or to

a combination of these events. This problem may likely be resolved by X-ray diffraction, just as it has recently been possible to confirm the postulated difference in structure of oxy- and reduced hemoglobin in this manner (Perutz, 1964).

Clearly, such interpretations are tentative and must be viewed with caution. Recently, conformational changes have been invoked frequently to explain observed variations in the properties of proteins consequent to their interactions with smaller molecules such as substrates, cofactors and prosthetic groups, and metal atoms. Physicochemical evidence for these structural alterations is not always abundant and such conclusions are tenuous, of course, when based solely upon minor changes in optical rotatory dispersion in the longer-wavelength regions of the spectrum. Cotton effects at shorter wavelengths may underlie rotational alterations observed in the visible spectrum and several instances of this type have been recognized already (Ulmer and Vallee, 1965). Interpretations are especially difficult in heme proteins where the pronounced rotatory power of many overlapping absorption bands may influence rotatory dispersion over wide ranges of the spectrum and at wavelengths far removed from the maxima of the optically active chromophores. Thus at this time it remains uncertain to what extent the small increase in magnitude of the intrinsic Cotton effect upon reduction of cytochrome *c* arises from changes in protein helicity or, alternatively, may reflect the pronounced changes in rotatory dispersion at longer wavelengths. Moreover, the presence of unrecognized optically active metal-protein ligand absorption bands near 233 mμ (Mulay and Selwood, 1955) cannot now be eliminated with certainty. Analogous problems in evaluation of the rotatory dispersion changes in ferri- and ferromyoglobin and myoglobin complexes have been discussed by Samejima and Yang (1964).

It has long been accepted that the subcellular organization of proteins importantly affects their biological activities. Thus the recognition in recent years that phospholipids may be implicated in the function of cytochrome *c* (Reich and Wanio, 1961a; Ball and Joel, 1962; Wharton and Griffiths, 1962; Green and Fleischer, 1963) seems especially pertinent to studies of the structure of this protein. Recently, isooctane-soluble cytochrome *c*–phospholipid complexes have been described (Reich and Wanio, 1961b; Das and Crane, 1964) presumably involving association between negatively charged phospholipids and positive free amino groups of the protein. Since such complexes may possibly simulate the physical environment of cytochromes in the mitochondrion, the effect of complex formation on protein conformation was examined by means of optical rotatory dispersion.

The details of the Cotton effect curves of the lipid-protein complexes in isooctane are virtually identical to those of the aqueous solution of protein (Figure 5). Moreover, the magnitude of the trough of the negative Cotton effect at 233 mμ is unaltered by the formation of the cytochrome *c*–phospholipid complex, suggesting that the structure of the protein and its lipid complex are identical at least within the limits of detection by spectropolarimetry.

Neither the protein nor the phospholipid alone can be extracted into isooctane but their complex is quite soluble in this solvent (Das and Crane, 1964). Moreover, the complex is stable for several days at room temperature. Hence it appears that the phospholipid stabilizes the protein against this strongly hydrophobic medium without apparent alteration in the physical structure of the cytochrome. If such a complex does, indeed, have similarities to the native state of cytochrome *c* such stabilization may bear upon the manner in which protein function is preserved in a nonaqueous region of the cellular environment (D. D. Ulmer, B. L. Vallee, A. Gorchein, and A. Neuberger, paper submitted for publication).

As noted in the present study, commercially available cytochrome *c* is largely in the oxidized form (Figure 5). While some changes in the dispersion curve result from complete oxidation with ferricyanide, particularly an increase in magnitude of the positive Cotton effect at the Soret band (Figure 1), these alterations are not nearly so marked as those resulting from complete reduction of the protein (Figure 2). These comments are pertinent to the apparent difference between the present observations and those recently reported by Shashoua (1964), who found much less pronounced changes in optical rotatory dispersion to accompany oxidation-reduction of cytochrome *c* than are here reported. Based upon the known molar absorptivity, $\epsilon_{550}^{1\text{ cm}}$, of cytochrome *c*, it appears that the data he reports were obtained upon material which was only partially reduced. The striking Cotton effect at the α band, observed in the presence of a magnetic field, might be still more intense if the fully reduced protein were examined by this novel method.

The prominent Cotton effects residing in the spectral range 275–290 mμ indicate that cytochrome *c* endows the chromophores of the aromatic amino acids with an unusually high degree of asymmetry, perhaps through stacking of the rings in some areas of the chain (Fasman *et al.*, 1964). The disappearance of one Cotton effect upon reduction of the protein is of special interest. Such a phenomenon might be another manifestation of altered protein structure. On the other hand, such a change could reflect the direct interaction of an aromatic chromophore with the asymmetric environment controlled by the heme iron atom. These and other alternatives are currently being investigated.[2]

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Ball, E. G., and Joel, C. D. (1962), *Intern. Rev. Cytol. 13*, 99.

Beychok, S., and Blout, E. R. (1961), *J. Mol. Biol. 3*, 769.

[2] The author gratefully acknowledges the support and encouragement of Dr. Bert L. Vallee during the course of these investigations.

Blauer, G., and Ehrenberg, A. (1963), *Acta Chem. Scand. 17*, 8.
Blout, E. R. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., ed., New York, McGraw-Hill, p. 238.
Blout, E. R. (1964), *Biopolymers, Symp. No. 1*, 397.
Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.
Breslow, E., Beychok, S., Hardman, K. D., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 304.
Das, M. L., and Crane, F. L. (1964), *Biochemistry 3*, 696.
Ehrenberg, A., and Theorell, H. (1955), *Acta Chem. Scand. 9*, 1193.
Eichhorn, G. (1961), *Tetrahedron 13*, 208.
Eichhorn, G., and Cairns, J. F. (1958), *Nature 181*, 994.
Fasman, G. D., Bodenheimer, E., and Lindblow, C. (1964), *Biochemistry 3*, 1665.
Fridovich, I. (1962), *J. Biol. Chem. 237*, 584.
George, P. (1961), *in* Haematin Enzymes, part 1, Lemberg, J. E., and Morton, R. K., eds., London, Pergamon, p. 18.
George, P., and Glauser, S. C. (1959), Abstracts of the Third Meeting of the Biophysical Society, Pittsburgh, p. D. 4.
Green, D. E., and Fleischer, S. (1963), *Biochim. Biophys. Acta 70*, 554.
Hagihara, B., Sekuzu, I., Tagawa, K., Yoneda, M., and Okunuki, K. (1958), *Nature 181*, 1588.
Harrison, S. C., and Blout, E. R. (1965), *J. Biol. Chem. 240*, 299.
Li, T. K., and Vallee, B. L. (1964), *Biochemistry 3*, 869.
Margoliash, E., and Frohwirt, N. (1959), *Biochem. J. 71*, 570.
Mulay, L. N., and Selwood, P. W. (1955), *J. Am. Chem. Soc. 77*, 2693.
Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K. (1958), *J. Biochem. 45*, 815.
Perutz, M. F. (1964), *Nature 203*, 687.
Reich, M., and Wanio, W. W. (1961a), *J. Biol. Chem. 236*, 3067.
Reich, M., and Wanio, W. W. (1961b), *J. Biol. Chem. 236*, 3058.
Rodkey, F. L., and Ball, E. (1950), *J. Biol. Chem. 182*, 17.
Samejima, T., and Yang, J. T. (1964), *J. Mol. Biol. 8*, 863.
Schellman, J. A., and Schellman, C. (1964), *Proteins 2*, 1.
Shashoua, V. E. (1964), *Nature 203*, 972.
Sund, H., and Theorell, H. (1963), *Enzymes 7*, 25.
Ulmer, D. D., and Vallee, B. L. (1963), *Biochemistry 2*, 1335.
Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.
Urnes, P., and Doty, P. (1961), *Advan. Protein. Chem. 16*, 401.
Urnes, P. J., Imahori, K., and Doty, P. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1635.
Wharton, D. C., and Griffiths, D. E. (1962), *Arch. Biochem. Biophys. 96*, 103.
Widmer, C., and Crane, F. L. (1958), *Biochim. Biophys. Acta 27*, 203.
Yang, J. T., and Samejima, T. (1963), *J. Biol. Chem. 238*, 3262.
Yonetani, T., and Theorell, H. (1962), *Arch. Biochem. Biophys. 99*, 433.