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Optical Rotatory Dispersion of Phycocyanin*

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ABSTRACT: The optical rotatory dispersion (ORD) (700–220 mμ) of phycocyanin isolated from the blue-green algae *Phormidium luridum*, *Plectonema calothricoides*, and *Synechococcus lividus* has been measured. ORD curves in the visible show multiple Cotton effects for the chromophore absorptions at ~620 mμ (+) and ~350 mμ (−). The ultraviolet ORD curves show a trough at 231 mμ with a crossover point at 222 mμ; the $[\alpha]_{231}$ of −3900 ± 200° indicates the protein to have a small amount of α-helix conformation. Urea denaturation irreversibly destroys the optical activity of the chromophore absorptions. The ORD behavior of phycocyanins from *Ph. luridum* and *P. calothricoides* resembles each other closely, while *S. lividus* phycocyanins are substantially different. The ORD curves of pairs of protio- and deuteriophycocyanins are essentially identical in both H_2O and D_2O. The rotatory dispersion of phycocyanin appears to be independent of the state of aggregation of the protein.

Phycocyanin, an important member of the class of biliproteins, is a blue, photosynthetic pigment found widely distributed in blue-green algae. Although a number of physical–chemical studies of the chromoprotein have been carried out, its detailed structure is largely unknown. The quaternary structure of phycocyanin involves a monomer, trimer, and hexamer equilibrium, which has been deduced from a study of the aggregation behavior of the protein (Hattori *et al.*, 1965b; Scott and Berns, 1965). Some additional information about the tertiary and secondary structure of phycocyanin can be deduced from thermal denaturation experiments (Hattori *et al.*, 1965a). This paper reports some new information about phycocyanin based on data obtained from the measurement of the ORD[1] properties of the protein in the native and in the urea-denatured state.

Experimental Section

Protein Preparation. The protio- and deuteriophycocyanins[2] from *Phormidium luridum*, *Plectonema calothricoides*, and *Synechococcus lividus* were isolated

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[1] Abbreviations used: ORD, optical rotary dispersion; CD, circular dichroism.

[2] The prefix "deuterio" refers to phycocyanin containing 99.6% deuterium at all nonexchangeable positions. The prefix "protio" refers to ordinary phycocyanin with hydrogen of mass 1 at all nonexchangeable positions.

and purified as previously described (Hattori *et al.*, 1965a). All protein samples studied had a purity ratio, 620:280-mμ absorption, of 4.4:4.6. Before use, samples were dissolved in sodium phosphate solution (prepared with H_2O or D_2O) and dialyzed against the buffer of the desired composition (either 0.01 M sodium phosphate, pH 7.5, 7.0, 6.5, and 6.0; or 0.02 M sodium acetate, pH 5.3) for 1 day at ~4° with at least three changes of external solutions. Urea denaturation experiments were carried out by dialyzing a solution of phycocyanin (0.22 mg/ml in 0.01 M phosphate buffer, pH 6.0) against a 2, 4, 6, or 8 M urea solution (pH adjusted to 6.0 by the addition of a small amount of phosphoric acid) at ~4° for 1 day with three changes of external solution. The hydrogen ion concentration (pH) was determined by a Beckman Model 76 pH meter. The corresponding values in D_2O buffers (pD) were estimated by addition of 0.4 unit to the reading of the pH meter (Mikkelson and Nielson, 1960).

Spectral Measurements. Absorption spectra were measured with a Cary Model 14 recording spectrophotometer. The phycocyanin concentration was estimated by taking $E_{1cm}^{0.1\%}$ 7 for the extinction of the peak near 620 mμ for both protio- and deuteriophycocyanin in H_2O or D_2O at pH 7.0, 0.01 M sodium phosphate buffer.

Rotatory dispersion and circular dichroism curves were determined with a Durrum-Jasco ORD/UV-5 optical rotatory dispersion recorder. Measurements were made on solutions in cylindrical, sealed quartz cells, 10-mm path length, in the 700–550- and 300–210-mμ range, and in 50-mm cells in 550–300-mμ range. For more dilute solutions, the 20- or 50-mm path length cell was used; conversely, for the more concentrated solutions, a short path length cell, 5, 2, or 1 mm, was used. In this way, the optical density of the solutions was never greater than 1.8 ODU (and more commonly around 1.6). The base line was recorded using the appropriate blank solution in the cell. Duplicate and replicate rotatory dispersion measurements were reproducible to ±0.003°. The observed rotations had a maximum value of 0.080–0.100° at 231 mμ, and were generally in the range 0.020–0.050° in the visible. The wavelength could be reproduced to ±2 mμ from 700 to 300 mμ and to ±1 mμ elsewhere. With the intense absorption bands of the phycocyanin solutions, the presence of artifacts in the observed rotatory dispersion curves is always possible.[3] The ratio of stray light to monochromatic light seems to be always small since the shape and magnitude of the Cotton effects are independent of the solution concentration and cell path length. Another consideration is the fluorescence properties of phycocyanin. Only the 620-mμ band shows fluorescence so we assume that the region below 500 mμ is not affected by the fluorescence. On the other hand the fluorescence of the 620-mμ band considerably increases the noise level in the 600–640-mμ region. Consequently, the crossover placement of the blue band Cotton effect is difficult (uncertainty ±5 mμ). At any rate the uncertainty in the optical rotations caused by the fluorescence in this region is still about the same as that caused by the errors in the preparation and determination of the concentration of the sample, as seen in the other regions of the spectrum. Infrared spectra of protio- and deuterio-*Ph. luridum* phycocyanin were determined in the solid state (potassium bromide pellets) with a Beckman IR-7 spectrophotometer.

Results

Native Protein. The rotatory dispersion curves, from 700 to 220 mμ, of protiophycocyanin isolated from *S. lividus*, *P. calothricoides*, and *Ph. luridum*, with their corresponding absorption spectra, are shown in Figure 1. The electronic spectra of the three proteins are nearly identical and show intense absorption peaks at 612, 616, and 618 mμ (red band) and relatively weak absorptions at ~355, ~358, and ~355 mμ (blue band), respectively. In the ultraviolet region, the spectra show a band at 278 mμ, as well as extremely strong absorption below 240 mμ. The red and blue bands arise from chromophore absorption, while the band at 278 mμ arises from the aromatic side chains in the protein. The band further into the ultraviolet arises from the polypeptide transitions. The ORD curves show positive Cotton effects at the red band absorption with an inflection point at ~594, ~626, and ~626 mμ, and negative Cotton effects at the blue band absorption with an inflection point at 334, 340, and 340 mμ. The trough of an intense negative Cotton effect, associated with the protein absorption, appears at 231 mμ with a crossover point at 222 mμ. The shapes and amplitude of the ORD curves are almost identical for *P. calothricoides* and *Ph. luridum* phycocyanin. On the other hand, the *S. lividus* phycocyanin ORD curve shows a substantial difference from the others in the shape of the red band Cotton effect. Further, a 32-mμ blue shift of the inflection point is also observed in this case, and the magnitude of the *S. lividus* phycocyanin ORD curve in the 300–550-mμ region is greater than for the other phycocyanins. Circular dichroism spectra of *Ph. luridum* phycocyanin (pH 7.0, 0.01 M phosphate buffer, 0.22 mg/ml) showed a positive Cotton effect centered at about 610 mμ and a negative Cotton effect centered at about 360 mμ.

The optical activity of the three protio- and deuteriophycocyanins, in H_2O and D_2O, was measured (standard condition, 0.23 mg/ml in pH (pD) 7.0, 0.01 M phosphate buffer). For the protein Cotton effect, the position of the trough is at 231 mμ, and the crossover point is at 222 mμ for all the proteins studied. The specific rotation for the proteins is independent (within experimental error) of the algal source, isotopic substitution, and solvent. The values of $[\alpha]_{231}$ range from −3700

[3] This possibility is especially important in the visible region of the spectrum. However, a careful assessment of the experimental conditions used in our work indicates that the observed effects are real.

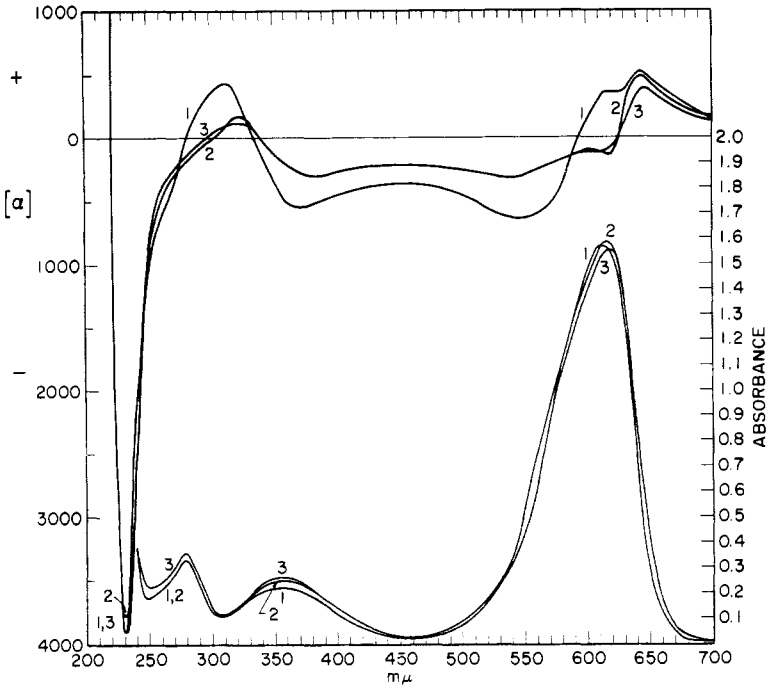

FIGURE 1: Optical rotatory dispersion and absorption spectra of phycocyanin from various algae: (1) *S. lividus;* (2) *P. calothricoides;* (3) *Ph. luridum* (0.22 mg/ml in pH 7.0, 0.01 M phosphate buffer).

to $-3900 \pm 200°$. The shape and magnitude of the ORD curves for the different phycocyanins in the protio and deuterio forms, in H_2O or D_2O, are very nearly identical. However, a blue shift of the red band inflection point of ~6 and ~12 mμ from the value for the protioproteins is noted for the deuterioproteins of *Ph. luridum* and *P. calothricoides*. Similar shifts are seen in the absorption spectra (Hattori *et al.*, 1965a). No measurable shifts are observed for the red band inflection point of deuteriophycocyanin from *S. lividus* nor for the blue band inflection point for any of the proteins.

The optical rotatory dispersion of *Ph. luridum* protiophycocyanin was determined under a variety of conditions. Measurements were made on protein solutions at different pH values (5.3, 6.0, 6.5, 7.0, and 7.5), different ionic strengths (0.01, 0.20, and 0.40 M phosphate, at both pH 6.0 and 6.5), and different concentrations of protein (0.05, 0.11, 0.21, 0.38, 1.05, and 1.84 mg/ml at pH 5.3). The protein optical activity appears to be independent of the pH, ionic strength, and concentration of protein in solution for the range of values studied. The position of the trough is at 231 mμ and the crossover point is at 222 mμ. The values of $[\alpha]_{231}$ center around $-3900 \pm 200°$. The shape and magnitude of all the ORD curves appear to be very nearly the same. On the other hand, slight shifts of the red band inflection point are observed. Corresponding shifts are apparent in the absorption spectra for different states of aggregation (Hattori *et al.*, 1965b).

Urea Denaturation. The optical activity and electronic spectra of *Ph. luridum* protiophycocyanin in 2, 4, 6, or 8 M urea solution at pH 6.0 are shown in Figure 2.[4] By comparison to the native protein, the intensity of the red band absorption (and fluorescence) is drastically reduced in 2 M urea solution. With increase in urea concentration, the spectra show some change in going from 2 to 4 M urea, but no further change is noted as the urea concentration is increased to 8 M. The intensity of the blue band and the 278-mμ band, however, gradually increases as the concentration of urea is increased. The magnitudes of the red and blue band Cotton effects are measurably reduced even in 2 M urea solution. No optical activity is associated with the red and blue bands for phycocyanin in 4, 6, and 8 M urea at pH 6. Similar measurements were made at pH 8–9. Although the native protein is unstable in this pH range, the red and blue absorption bands retain some optical activity even in 8 M urea solution.

The protein optical activity is also changed in urea solution. In 2 M urea, the value of $[\alpha]_{231}$ is decreased to ~1200° and the crossover point is shifted to 219 mμ. As the concentration of urea increases from 2 to 8 M, the inflection point disappears, and the trough shifts to shorter wavelength (~225 mμ) while increasing in magnitude (−1900°). Removal of the urea by dialyzing the solution against pH 6.0 phosphate buffer does not fully renature the protein (Figure 2). The protein optical activity is partially restored, $[\alpha]_{231}$ −2300° (or about 60% of the native protein values), if all the operations are carried out in the cold (~4°). The red and blue band Cotton effects are likewise only partially restored by this operation. If the cold, 8 M

[4] The protein exhibits its greatest stability at about pH 6.0 (H. L. Crespi, unpublished data).

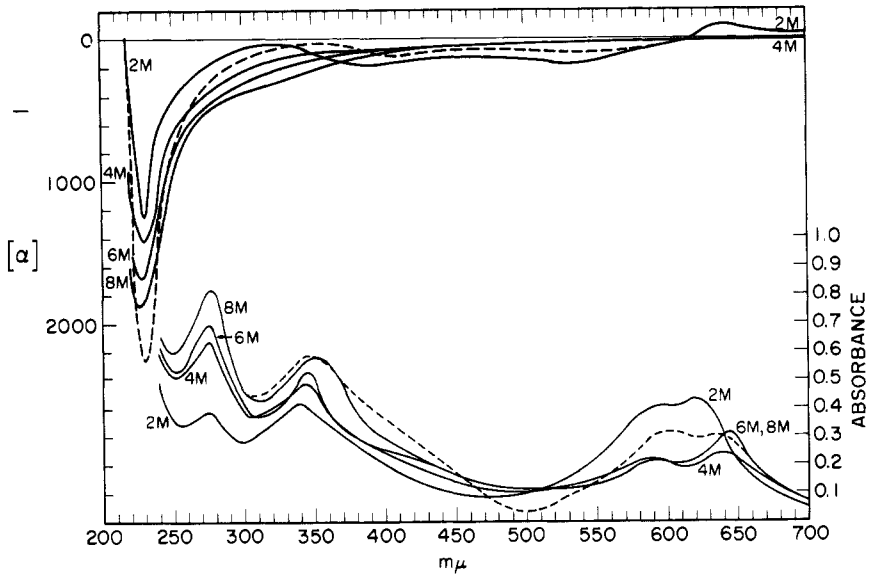

FIGURE 2: Optical rotatory dispersion and absorption spectra of *Ph. luridum* phycocyanin: solid line, 0.22 mg/ml in 2, 4, 6, and 8 M urea at pH 6.0; dashed line, pH 6.0, 0.01 M phosphate buffer after removal of 8 M urea.

urea solutions are exposed to room temperature (~22°) for extended periods of time before removal of the urea, the ORD and absorption spectra are unchanged from those of the denatured protein. The ORD curves and absorption spectra of *S. lividus* and *P. calothricoides* protiophycocyanin in 8 M urea solution (pH 6.0) are similar in shape to those of *Ph. luridum* phycocyanin. The rotatory dispersion shows a very slight optical activity at the red and blue bands as well as a broad trough in the 220–230-mμ region. The magnitude of the trough (−1300°) for *S. lividus* phycocyanin is somewhat less than that of the *Ph. luridum* and *P. calothricoides* phycocyanin. This value corresponds to that of *Ph. luridum* in ~4 M urea.

The infrared spectra of protio- and deuterio-*Ph. luridum* phycocyanin show strong absorption at 1650 and 1532 cm^{-1}. There are no other prominent absorptions or shoulders near the 1650-cm^{-1} band.

Discussion

Protein. The ORD curves of phycocyanin do not yield the usual protein parameters. The fluorescence and intense color, with concomitant low rotations, preclude precise measurements above 300 mμ. In addition, the red and blue band Cotton effects overlap with the Drude curve for the protein. As a result, a meaningful analysis of the protein optical activity in the visible region is not possible and, consequently, the Moffitt constants were not determined. Moreover, instrument limitations and solute absorption prevented the extension of the rotatory dispersion curves to much below 220 mμ. Fortunately, the optical rotation in the 240–220-mμ region is particularly dependent on the conformation of the protein. In fact, a trough of a negative Cotton effect at ~233 mμ has been related to the α-helix content of proteins and polypeptides (Simmons *et al.*, 1961).

The ORD curve of phycocyanin contains a trough at 231 mμ with [α] −3900° and a crossover point of 222 mμ. This we take to indicate the presence of a small amount of right-handed, α-helix conformation in the phycocyanin protein. The slight blue shift (3 mμ) of the crossover point may arise from the superposition of the ORD properties of the α helix and non-α helix portions of the protein ("random coil") (Jirgensons, 1966). Considering the paucity of data and the difficulties inherent in such an estimate, it is unwise to make a quantitative estimate of the α-helix content of phycocyanin (Carver *et al.*, 1966). The value of $[\alpha]_{231}$ is similar to the values found for some other globular proteins (Jirgensons, 1965).

All the phycocyanins (protio and deuterio) studied here have approximately the same α-helix content (same $[\alpha]_{231}$) and probably have about the same secondary and tertiary structure. A similar conclusion was reached in a recent immunochemical study (Berns, 1963). Protio- and deuteriophycocyanins in either H_2O or D_2O likewise show about the same α-helix content. Primary and secondary isotope effects have been observed in studies of the thermal denaturation of phycocyanin; the deuterioprotein is less stable than the protioprotein, and the protein is less stable in H_2O than in D_2O (Hattori *et al.*, 1965a). The failure to observe a deuterium isotope effect for the conformation of the protein may be due to the insensitivity of the measurement and to the low α-helix content in the native structure of the protein. The static isotope effect on the equilibrium configuration may in fact be

small, but the dynamic isotope effect on the rate of denaturation should be measurable (Maybury and Katz, 1956).

Phycocyanin has previously been described as a system of reversibly interacting components. At low pH (~5.3), high protein concentration, or high ionic strength, the hexamer predominates; at moderate pH (~7.0), moderate protein concentration, or low ionic strength, the trimer predominates; and at low ionic strength and low protein concentration (<0.01%), the monomer predominates (Hattori *et al.*, 1965b). The intensity, and to a lesser extent the position, of the red band absorption is sensitive to the state of aggregation of phycocyanin. On the other hand, the optical activity of phycocyanin is not sensitive to its state of aggregation, since it is independent of pH, ionic strength, and protein concentration. There may, of course, be small differences which are within the experimental error inherent in the measurement. To a first approximation, the α-helix content of the phycocyanin, and presumably the secondary structure of the protein, appears to be the same for the monomer, trimer, and hexamer. Native phycocyanin, then, appears to behave as compact, rigid particles in a fashion typical of globular proteins (Urnes and Doty, 1961).

The urea denaturation of phycocyanin presumably involves an α-helix–random coil transition. The absorption spectra of *Ph. luridum* phycocyanin show a progressive increase in the intensity of the 278-mμ band as the urea concentration is increased from 2 to 8 M. This change in the aromatic side-chain absorption can be related to an increase in the extent of denaturation (unfolding) of the protein (Beaven, 1961). The ORD curves should likewise show, with increase in urea concentration, a progressive decrease in the amplitude of the trough in the 230-mμ region until the minimal value for the random coil conformation of the protein is reached. The rotatory dispersion curve for the phycocyanin in 8 M urea solution does, in fact, show the trough shifted to ~225 mμ and its amplitude ~1900°. This value is typical of a denatured protein predominantly in the random coil conformation (Jirgensons, 1965). In 2 M urea solution, however, the crossover point is shifted to 219 mμ, and a trough at 231 mμ (~1200°) is still evident. As the urea concentration increases, the position of the trough shifts to shorter wavelength and the amplitude increases. This behavior is not consistent with a simple α-helix–random coil transition if only these two species are assumed to give rise to optical activity in the 220–240-mμ region. The data can, however, be rationalized by assuming that some other structure in the native protein is contributing to the rotation at 220–300 mμ. This added optical activity may then be less easily lost in urea solution than that of the α helix.

There are several possible sources of the added optical activity. One is from some protein conformation other than the right-handed α helix and the random coil. A conformation that has been previously suggested for globular proteins is the β conformation. The responses of the β- and α-helix conformation to urea denaturation are different (Callaghan and Martin, 1963), thus supporting this explanation. The difference in the position of the trough and crossover point for native phycocyanin, at 231 and 222 mμ, and that usually observed for α-helix-containing proteins (233 and 223 mμ) may be further evidence for the presence of an amount of the β conformation in phycocyanin (Davidson *et al.*, 1966). Arguing against this, however, is the observation that the infrared spectrum of solid phycocyanin only shows an absorption band at 1650 cm^{-1} which is typical of the α conformation. No prominent absorption band or shoulder appears at ~1630 cm^{-1}, the position typical of the β conformation (Krimm, 1962). Admittedly, small amounts of the β conformation would not be observed by this method. A second source of added optical activity may be from the chromophore absorption bands. This seem unlikely since urea rapidly destroys its optical activity even at low urea concentration (see below). A third source of optical activity may be from side-chain optical activity. The low content of cystine in the phycocyanin protein (Berns *et al.*, 1963) rules out appreciable contribution to the optical activity from the disulfide bonds. On the other hand, there are a substantial number of aromatic groups in the protein (as evidenced by the absorption band at 278 mμ). Aromatic side-chain optical activity, in poly-L-tyrosine, cancels part of the larger rotational strength of the α helix and shifts the trough from 236 to 238 mμ (Beychok and Fasman, 1964). This shift, however, is opposite to that observed for phycocyanin. Another important point to consider is that the random coil conformation of the denatured protein may be optically different from the "random coil" portions of the native protein.

The response of the phycocyanin to urea denaturation can be adjudged from the protein optical activity changes with increasing urea concentration. From this point of view, *Ph. luridum* and *P. calothricoides* phycocyanin are quite comparable. On the other hand, *S. lividus* phycocyanin seems to be more resistant to urea denaturation, *i.e.*, its protein optical activity in 8 M urea is equivalent to that of the other phycocyanin in lower concentrations of urea (4–6 M urea). Greater resistance of the *S. lividus* phycocyanin to thermal denaturation as compared to other phycocyanins also has been observed. (Hattori *et al.*, 1965a). The different properties observed for *S. lividus* phycocyanin presumably arise from major differences in amino acid sequence.

Chromophore. The molecular structure of the phycocyanin chromophore group has not been established. However, it has been postulated to be a linear tetrapyrrole derivative similar to the bile pigments (Gray, 1953). The prosthetic group was first claimed to be a bilidiene of the mesobiliviolin type (Lemberg and Bader, 1933). It was later shown, however, that this substance must be an artifact produced during the isolation procedure (Ó hEocha, 1958a,b). Phycocyanobilin, the chromophore, has not been crystallized,

and what is known about it is, for the most part, based on spectral studies (Ó hEocha, 1963). It is alleged to have absorption maxima at 612 and 360 mμ in chloroform. These lie between the expected positions for a normal bilidiene and a bilitriene. The chromophore group absorption spectrum in native phycocyanin shows a maximum at 620–615 mμ, which is asymmetric to the short-wavelength side, and a broad maximum at 360–350 mμ. Thermally denatured phycocyanin shows similar spectra except the intensity of the red band is greatly decreased (with concomitant increase in the short-wavelength asymmetry) (Hattori *et al.*, 1965a). Finally, the fluorescence excitation spectrum of phycocyanin displays two bands, at 625 and 595 mμ, and a broad band at 370 mμ (Berns *et al.*, 1963).

The presence of Cotton effects in the rotatory dispersion curves of phycocyanin at the red and blue absorption bands shows that with high probability the phycocyanobilin attached to the protein is optically active. The magnitude of the red band Cotton effect, based on ~5% by weight phycocyanobilin in the protein (Brody and Brody, 1961), is about 10,000°. The optical activity of phycocyanobilin is consistent with the presence of an inherently dissymmetric chromophore in the molecule. The bile pigment, *d*-urobilin, has been shown to possess optical activity of this kind (Moscowitz *et al.*, 1964). The optical activity of the chromophore can, however, arise in a different way. When a symmetric molecule is bound to a protein in the α-helix conformation, its absorption bands are optically active (Stryer and Blout, 1961). Conversely, when the protein is denatured, the chromophore is no longer optically active. Finally, inherent optical activity of the chromophore may also be enhanced by association with the ordered protein. Measurement of the rotatory dispersion properties of authentic phycocyanobilin will be needed to make a choice between these alternatives.

The rotatory dispersion curve of phycocyanin in the region of the red band can be interpreted as the sum of two positive Cotton effects, one with an inflection point at ~620 mμ and the other with an inflection point at ~590 mμ. The close proximity of the blue band Cotton effect to that of the protein does not allow any judgment to be made as to the multiplicity of that Cotton effect. However, circular dichroism spectra show a definite splitting of the CD absorption bands at both the red (620 and 590 mμ) and blue (370 and 345 mμ) bands of phycocyanin. The multiplicity of the Cotton effect can be more readily observed with *S. lividus* phycocyanin than with the other phycocyanins. The large difference, in the visible region ORD curves of the *S. lividus* phycocyanin as compared to the *Ph. luridum* and *P. calothricoides* phycocyanin are in contrast to the relatively small differences in the absorption spectra and protein optical activity.

The multiplicity of the Cotton effects, the distinct doublet in the fluorescence excitation spectrum, and the asymmetric absorption bands may be taken as evidence for the existence of two absorbing species in the protein, *i.e.*, one at ~590 mμ and the other at ~620 mμ.[5] There is considerable evidence that there is only one kind of chromophoric group in phycocyanin, regardless of algal source (Ó hEocha, 1963). The two absorbing species could then represent phycocyanobilin attached to different portions of the protein. The mode of attachment of the prosthetic group to the protein is at present entirely unknown.

The electronic spectra of *Ph. luridum* phycocyanin in 2, 4, 6, or 8 M urea solution at pH 6.0 (Figure 2) show two absorption bands between 550 and 650 mμ with one peak always appearing at ~590 mμ. The second band seems to shift progressively from 620 to 645 mμ as the urea concentration increases. By contrast, in 4, 6, or 8 M urea solution at pH 8–9, the major absorption is at ~590 mμ with only a shoulder in the region of 625 mμ. The removal of the urea does not fully reestablish the native protein electronic spectrum in the visible region. This indicates that the chromophore group is undergoing alteration on treatment with urea. The ~590-mμ absorption band appears to be present in both the native and denatured protein. It seems likely that long-wavelength (~645 mμ) absorption for the denatured protein arises from an artifact formed from the chromophore group. Spectral changes occurring on urea denaturation of the other phycocyanins are also consistent with this argument. The loss of chromophore optical activity in urea solution is probably a consequence of both the destruction of the ordered structure of the protein and the alteration of the chromophore. The latter point is supported by the observation that removal of urea only partially restores the chromophore optical activity.

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[5] However, the alternate assignment of the 590- and 620-mμ absorption to different electronic transitions in the same molecule of course, would also satisfy the data.

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