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Association and Dissociation of Phycocyanin and the Effects of Deuterium Substitution on the Processes*

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ABSTRACT: Association-dissociation reactions of deuterio- and ordinary phycocyanin were followed by characteristic changes in the visible absorption spectra under various conditions of *p*H, ionic strength, and protein concentration. Light-absorption peaks of deuterio- and protiophycocyanin are as follows: 617, 625, and 621 mμ for the protio forms, and 610, 617, and 614 mμ for the deuterio forms.

Deuteration of the protein results in a shift of about 7 mμ toward shorter wavelength, irrespective of association state. Complementary information was obtained from sedimentation experiments. Phycocyanin associates or dissociates reversibly to form monomer, trimer, or hexamer, to an extent dependent on conditions. Under identical conditions, the association tendency of deuteriophycocyanin was found to be several times less than that of protiophycocyanin.

Svedberg was the first to show the occurrence of *p*H-dependent association and dissociation of phycocyanin and phycoerythrin (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929). This observation has been confirmed by subsequent investigations (Svedberg and Eriksson, 1932; Eriksson-Quensel, 1938; Hattori and Fujita, 1959). The most recent of these studies was carried out with samples isolated by improved methods, since some criticism was raised as to the purity of the samples used in earlier work. Unfortunately, most of the earlier studies were mainly concerned with the qualitative aspects of the association phenomena, and the quantitative aspects have not as yet been the subject of active investigation.

The recent success in culturing various algae and microorganisms in pure D_2O has provided a novel way to investigate the structure and function of biologically important substances by the use of fully deuterated compounds. Phycocyanin has been used as a model protein for the investigation of deuterium isotope effects on protein conformation (Berns *et al.*, 1962, 1963; Berns, 1963a,b; Hattori *et al.*, 1965). In one of these studies, we (Berns *et al.*, 1963) observed that phycocyanin does not obey Beer's law. Sedimentation behavior likewise suggested association behavior. Further, the precise positions of the absorption maxima of this protein are variable, depending on the source and even the method of preparation (Haxo and Ó hEocha, 1960; Ó hEocha, 1962). Bergeron (1963) has pointed out the correlation between absorption characteristics and association states, and association-dissociation phenomena appear to be a rather general feature of protein behavior (Reithel, 1963). A detailed knowledge of association-dissociation processes in phycocyanin thus becomes essential if we are to assess the effects of deuterium replacement on the structure and stability of phycocyanin.

In the work reported here, association in phycocyanin was followed by characteristic changes in absorption spectra. These data were correlated with sedimentation

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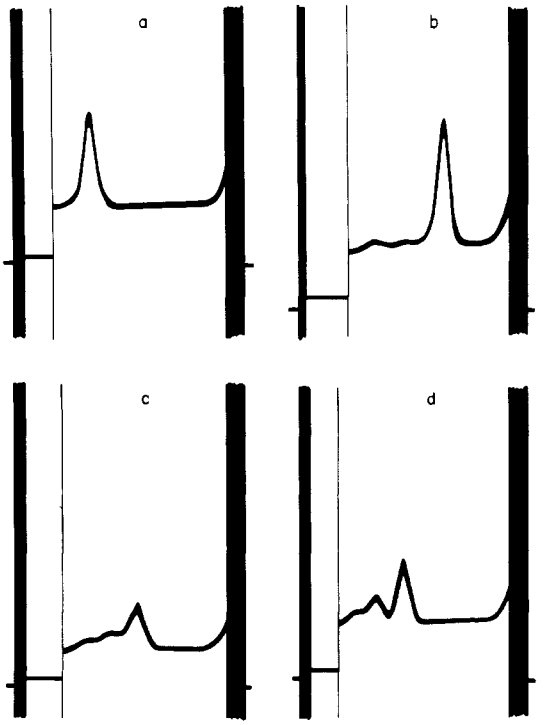

FIGURE 1: Sedimentation diagrams of phycocyanin. (a) deuteriophycocyanin at *p*H 7.0, 0.01 M phosphate buffer, 24 minutes after reaching maximum speed, 59,780 rpm; (b) deuteriophycocyanin at *p*H 5.3, 0.01 M phosphate buffer, 29 minutes after reaching maximum speed, 59,780 rpm; (c) protiophycocyanin at *p*H 5.3, 0.001 M phosphate buffer, 37 minutes after attaining maximum speed, 59,780 rpm; (d) deuteriophycocyanin at *p*H 7.0, 0.01 M phosphate buffer plus 0.2 M NaCl, 28 minutes after reaching maximum speed, 59,780 rpm.

experiments carried out under similar conditions. Comparative diffusion, partial specific volume, and viscosity measurements on protio- and deuteriophycocyanin were also made.

Within specified ranges of *p*H and ionic strength, phycocyanin exists either as monomer, trimer, or hexamer. Deuteration of the protein always favors dissociation. From the effects of changes in *p*H and ionic strength, it can be inferred that the forces holding the monomer units together in the associated structure are nonelectrostatic.

Materials and Methods

Protein Preparation. Phycocyanin was prepared from cells of the blue-green alga *Plectonema calothricoides* grown autotrophically in 99.6% D_2O or H_2O (DaBoll *et al.*, 1962). After partial fractionation by repeated ammonium sulfate precipitation (50% saturation), samples were further purified by adsorption on a DEAE-cellulose column equilibrated with 0.01 M sodium phosphate, *p*H 7.0, followed by elution with 0.1 M sodium phosphate, *p*H 7.0. This treatment removed allophycocyanin that is still present after ammonium sulfate fractionation. Purified samples of phycocyanin were dissolved in sodium phosphate buffers of various acidities and ionic strengths and dialyzed against the same buffers for 1 or 2 days, with an occasional change of external solutions. Concentrations of phycocyanin were determined from the weight of aliquots dried 24 hours at 105°. In sedimentation experiments, relative concentrations were estimated from the area of the schlieren pattern. The *p*H (and *p*D) of solutions was determined by a Beckman expanded-scale *p*H meter, Model 76.

Sedimentation Experiments. Sedimentation measurements were carried out in a Beckman-Spinco ultracentrifuge, Model E. Regular sectorial cells with a 1.2-cm light path were used, and the temperature of the rotor was maintained between 22 and 25°. The positions of the schlieren peaks in the sedimentation diagrams were read directly by a micrometer, and sedimentation coefficients were calculated from the slope of the usual semilogarithmic plot (Schachman, 1959). Since the amounts of minor components were found to be rather small under our conditions, no correction was made for the Johnston-Ogston effect. At very low phycocyanin concentrations, the light-absorption method was used. In these instances, the density of blackening of a photographic plate was measured with a double-beam recording microdensitometer, Joyce-Loebl and Company, Model MK IV B. The values of sedimentation coefficients were reduced to those in a standard state (20°; solvent, water).

Diffusion Experiments. Diffusion constants were estimated from the spreading of the boundary between solution and solvent, measured with a Perkin-Elmer electrophoretic apparatus, Model 18A, fitted with a high-pressure mercury arc lamp. The maximum height–area method was used.

Density Measurement and Apparent Specific Volume. Apparent specific volumes were calculated by standard methods from densities determined at 20° in a 5-ml pycnometer of the type described by Lipkin *et al.* (1944).

Viscosity Measurement. All determinations were carried out at 20.0° with Cannon-Fenske viscometers of 6-ml capacity. Kinematic energy terms were less than 5.0, flow times were about 400 seconds with pure water, and density terms were appropriate to the isotopic protein under study.

Spectra. Absorption and difference spectra were measured by a Cary spectrophotometer, Model 14R.

Results

Sedimentation. At *p*H 7.0 (phosphate buffer, 0.01 M), phycocyanin shows a single peak in the ultracentrifuge (Figure 1a). The sedimentation coefficients were about 7 for deuteriophycocyanin and about 6 for protiophycocyanin. Phycocyanin in this state is hereafter referred to as the S-6 component.

At *p*H 5.3 (phosphate buffer, 0.01 M) a faster-moving component appeared with a sedimentation coefficient of

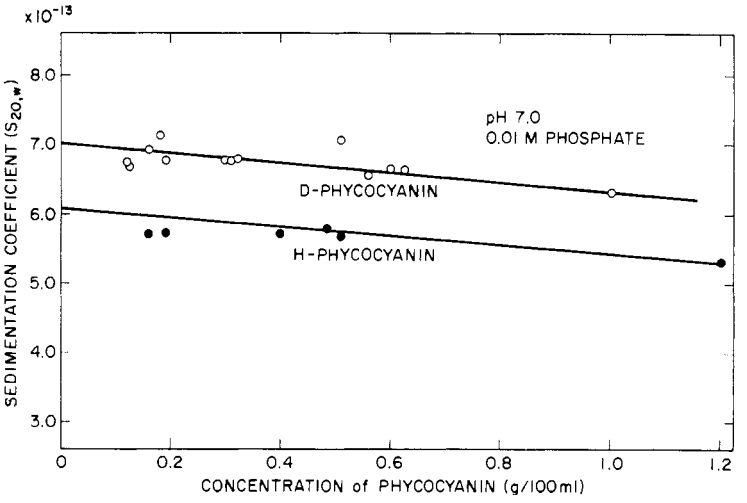

FIGURE 2: Concentration dependence of the sedimentation coefficient of phycocyanin at *p*H 7.0, 0.01 M phosphate buffer.

TABLE I: Sedimentation Coefficients of Deuteriophycocyanin under Various Conditions.

Solvent	*p*H	Concentration of Phycocyanin (mg/ml)	$s_{20,w} \times 10^{13}$ (sec^{-1})	Method
0.001 M phosphate	7.0	0.90	3.8	Light absorption
0.01 M phosphate	7.0	0.75	4.8	Light absorption
0.01 M phosphate	7.0		7.0[a]	Schlieren
0.01 M phosphate	7.0	3.2	6.7	Schlieren
0.01 M phosphate	7.0	6.0	6.9	Schlieren
0.01 M phosphate + 0.2 M NaCl	7.0	9.8	6.7,[b] 12.3[b]	Schlieren
0.001 M phosphate	5.3	0.65	3.8	Light absorption
0.01 M phosphate	5.3	0.56	4.8, 12.2	Light absorption
0.01 M phosphate	5.3		13.3[a]	
0.01 M phosphate	5.3	5.5	3.8,[c] 12.5	Schlieren
0.01 M phosphate	5.3	7.9	4.0,[c] 12.3	Schlieren

[a] Values from extrapolation to zero concentration. [b] S-6 about 30%; S-11 about 70%. [c] Minor component (less than 10%).

approximately 13 for deuteriophycocyanin and 11 for protiophycocyanin. This will be referred to as the S-11 component. The relative amount of the S-11 component increases with decreasing *p*H or with increasing ionic strength, and is accompanied by a decrease in S-6 component. At *p*H 5.3, 0.01 M phosphate, more than 90% of the phycocyanin exists as the S-11 component (Figure 1b). These observations are in good agreement with the results of earlier workers (Svedberg and Katsurai, 1929; Hattori and Fujita, 1959).

In addition to the S-6 and S-11 components, a component with a sedimentation coefficient of about 4 for deuteriophycocyanin and 3 for protiophycocyanin (see Figure 1b, c) can be observed. The amount of this component, however, was found to be relatively small (less than 5% in terms of peak areas in the sedimentation diagram) compared with the other two components in the *p*H range 7.2–5.0, and at the concentrations of phycocyanin used in these experiments (greater than 1.5 mg/ml). Decreasing the concentration of phycocyanin (to less than 1 mg/ml) or decreasing the concentration of the buffer increases the fraction of this slowest-moving component. This component may be a subunit of S-6.

On the other hand, if we increase the concentration of the buffer, or increase the ionic strength by addition of a

TABLE II: Sedimentation Coefficients of Protiophycocyanin under Various Conditions.

Solvent	*p*H	Concentration of Phycocyanin (mg/ml)	$s_{20,w} \times 10^{13}$ (sec^{-1})	Method
0.001 M phosphate	7.0	0.89	3.2	Light absorption
0.01 M phosphate	7.0	0.36	4.5	Light absorption
0.01 M phosphate	7.0	0.71	4.7	Light absorption
0.01 M phosphate	7.0		6.1[a]	Schlieren
0.01 M phosphate	7.0	1.5	5.7	Schlieren
0.01 M phosphate	7.0	6.7	5.7, 10.4[b]	Schlieren
0.01 M phosphate +0.2 M NaCl	7.0	1.2	5.8,[b] 9.9	Schlieren
0.001 M phosphate	5.3	0.33	2.8, 10.9	Light absorption
0.001 M phosphate	5.3	0.66	3.4, 10.5	Light absorption
0.01 M phosphate	5.3	0.80	3.5, 10.9	Light absorption
0.01 M phosphate	5.3		11.0[a]	Schlieren
0.01 M phosphate	5.3	6.7	2.9,[b] 10.2	Schlieren

[a] Values from extrapolation to zero concentration. [b] Minor component (less than 10%).

neutral salt such as sodium chloride, a marked increase in the fraction of S-11 (Figure 1d) occurs even at *p*H 7. This change of form of phycocyanin was found to be reversible. Below *p*H 5.2 and above *p*H 7.2 full reversibility becomes increasingly difficult.

The experimental conditions for the determination of the hydrodynamic characteristics were therefore specified as follows: (1) *p*H 7.0; 0.01 M phosphate; concentration of phycocyanin higher than 1.5 mg/ml; (2) *p*H 5.3; 0.01 M phosphate; concentration of phycocyanin higher than 1.5 mg/ml. Under condition (1), phycocyanin occurs practically entirely as the S-6 component, and under condition (2) as the S-11 component. Extrapolation to zero concentration further reduces errors due to the inevitable coexistence of small amounts of other components. Conductivity measurements on buffers with and without phycocyanin indicate that at *p*H 7.0, in 0.01 M phosphate buffer, less than 1% error in sedimentation coefficient is to be expected because of charge effects. At *p*H 5.3, 0.001 M phosphate, an error of 3–4% is probably introduced from this source.

Figure 2 shows the concentration dependence of the sedimentation coefficient of the S-6 component of deuterio- and protiophycocyanin. The S-11 component exhibited only slightly greater concentration dependence. The differences between sedimentation coefficient values for deuteriophycocyanin and for protiophycocyanin are about three times those expected solely on the basis of the mass increase due to deuterium substitution. A summary of sedimentation characteristics under a variety of conditions and extrapolation values at zero concentration is given in Tables I and II.

Some questions inevitably arise as to the effect of the association-dissociation reactions upon the sedimentation results. Data presented here indicate that at *p*H 7.0, 0.01 M phosphate buffer, and at moderate protein concentrations, the phycocyanin system is essentially a monomer-trimer system, $P_3 \rightleftarrows 3P$, with a dissociation constant of 10^{-2} g-mole/liter. At *p*H 5.3, 0.01 M phosphate, we have essentially a monomer-hexamer system, $P_6 \rightleftarrows 6P$, with a dissociation constant of 10^{-7} g-mole/liter. (These values are for protiophycocyanin; see Table VII.) Dilution experiments at *p*H 7.0 and 5.3 show that the velocity constants for both dissaggregation reactions are in the range of 10^{-3}–10^{-4} sec^{-1}. These rate parameters indicate that the observed sedimentation coefficients for the S-6 and S-11 components are the true sedimentation coefficients, with only negligible error introduced by aggregation interactions (see Gilbert, 1963; Gilbert and Jenkins, 1963). Further, the

TABLE III: Diffusion Constants of Phycocyanin.[a]

	Concentration[b]	*p*H	$D_{20,w}$ [c]
Deuterio-	0.16	7.0	4.12
protein	0.15	7.0	4.08
	0.18	5.3	3.86
Protio-	0.27	7.0	4.28
protein	0.15	7.0	4.39
	0.16	5.3	3.94
		7.0	4.3[d]
		5.0	4.0[e]
		7.0	4.2[f]

[a] Phosphate buffer, 0.01 M. [b] Units are g/100 ml. [c] Units are $cm^2/sec \times 10^7$. [d] Hattori and Fujita (1959). [e] Tiselius and Gross (1934). [f] Berns *et al.* (1964); his major phycocyanin component.

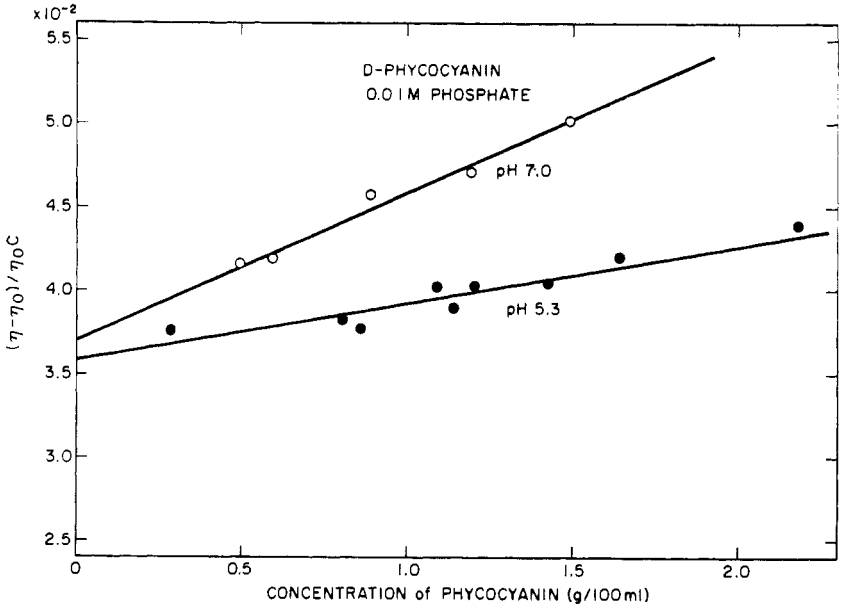

FIGURE 3: Viscosities of deuteriophycocyanin. Reduced kinematic viscosities $(\eta - \eta_0)/\eta_0 c$ are plotted against concentration, c; 0.01 M phosphate, 20.0°.

TABLE IV: Apparent Specific Volume of Phycocyanins.

Concentration (g/100 ml)	pH	Density (20°)[a] (g/ml)	Φ [b]
Deuterioprotein			
1.990	7.0	1.0053	0.701
1.990	7.0	1.0054	0.699
0.421	7.0	1.0036	0.699
2.808	5.3	1.0077	0.702
1.404	5.3	1.0035	0.703
0.936	5.3	1.0022	0.691
		Average:	0.699
Protioprotein			
4.097	7.0	1.0096	0.752
2.926	7.0	1.0068	0.746
2.049	7.0	1.0046	0.743
2.274	5.3	1.0052	0.743
1.706	5.3	1.0037	0.743
1.137	5.3	1.0023	0.739
		Average:	0.744

[a] Densities of solvents (0.01 M phosphate) at 20.0° were 0.99932 at *p*H 7.0 and 0.99930 at *p*H 5.3. [b] Apparent specific volume, Φ, is defined by the equation, $\Phi = [100(d_o - d)/cd + 1]/d_o$, where c is concentration of phycocyanin in g/100 mg, and d_o and d are densities of solvent and solution, respectively.

schlieren patterns exhibit only slight assymetry, and the area beneath the peaks remains constant. This gives additional assurance that the sedimentation calculations are meaningful (Singer and Campbell, 1952).

Diffusion. Table III shows the results of diffusion experiments carried out under the conditions specified. Since the diffusion constants of proteins are generally almost independent of concentration, no attempt was made to extrapolate to zero concentration. From Table III, the diffusion constants for protio- and deuteriophycocyanin are observed to differ by only a few per cent.

Partial Specific Volume. Apparent specific volumes of deuterio- and protiophycocyanin are given in Table IV. The partial specific volumes ($\bar{v}$) at *p*H 7.0 are identical with those at *p*H 5.3; in subsequent calculations the average values of $\bar{v}$ were used.

Viscosity. Viscosity data for deuteriophycocyanin in 0.01 M phosphate (*p*H 7.0 and 5.3) at 20.0° is shown in Figure 3. Protiophycocyanin yields similar plots. The differences in slopes observed at *p*H 7.0 and at *p*H 5.3 can be attributed to charge effects. Introduction of a large amount of salt to reduce the charge effect, a procedure usually adopted for viscosity measurements of proteins, cannot be employed here because of salt effects on the aggregation state of the phycocyanin. The intrinsic viscosities (Table V) for deuteriophycocyanin

TABLE V: Intrinsic Viscosity $[\eta]$[a] of Deuterio- and Protiophycocyanin.[b]

Deuteriophycocyanin		Protiophycocyanin	
*p*H 7.0	*p*H 5.3	*p*H 7.0	*p*H 5.3
0.0415	0.0388	0.0358	0.0336

[a] Intrinsic viscosities were calculated from the limiting kinematic viscosities according to the method of Tanford (1955). [b] Phosphate buffer, 0.01 M, 20.0°.

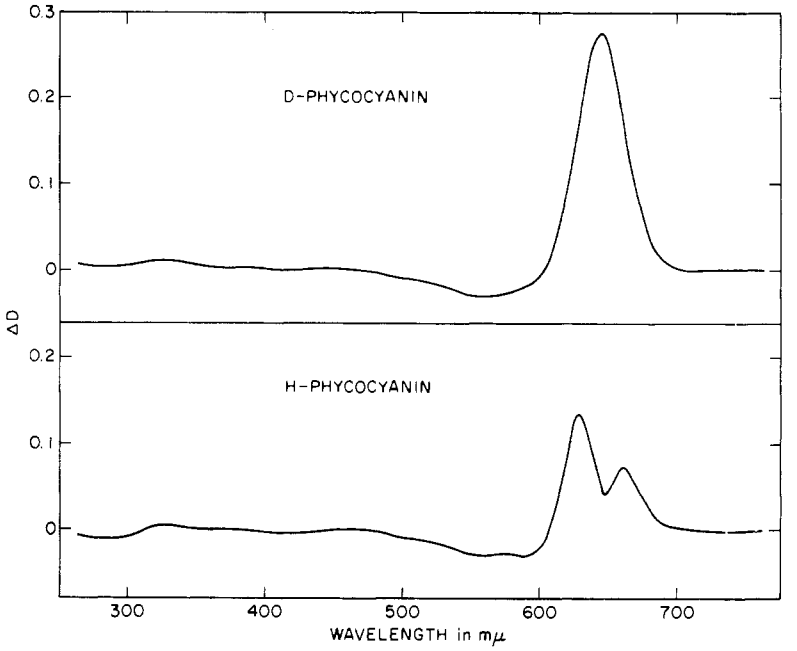

FIGURE 4: Difference spectra of phycocyanin in 0.01 M phosphate buffer (*p*H 5.4 and 7.2). Top, deuteriophycocyanin, 0.26 mg/ml; bottom, protiophycocyanin, 0.22 mg/ml. Reference: *p*H 7.2.

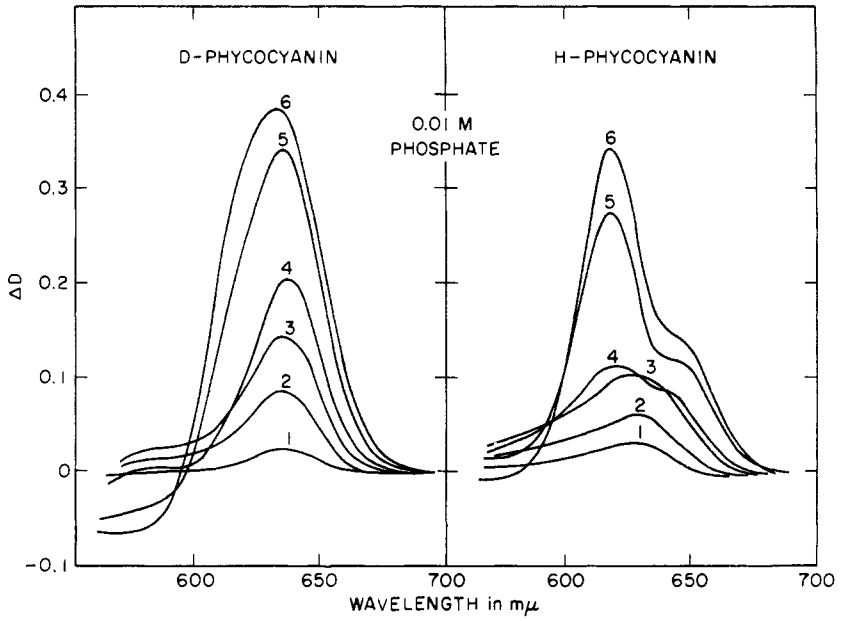

FIGURE 5: *p*H effect on the difference spectra of phycocyanin in 0.01 M phosphate. Left, deuteriophycocyanin, 0.27 mg/ml; right, protiophycocyanin. Curve 1, *p*H 7.2; curve 2, *p*H 7.0; curve 3, *p*H 6.6; curve 4, *p*H 6.3; curve 5, *p*H 5.6; and curve 6, *p*H 5.2. Reference: *p*H 7.3.

were found to be somewhat higher than those for protiophycocyanin, irrespective of the *p*H of the medium.

Molecular Weights. Table VI summarizes molecular weight data calculated from sedimentation coefficients, diffusion constants, and partial specific volumes. The large differences in the values of the sedimentation coefficients of deuterio- and protiophycocyanin (about 15% for S-6 and 20% for S-11) are compensated by an opposite change in the partial specific volume. The molecular weights for protiophycocyanin are identical,

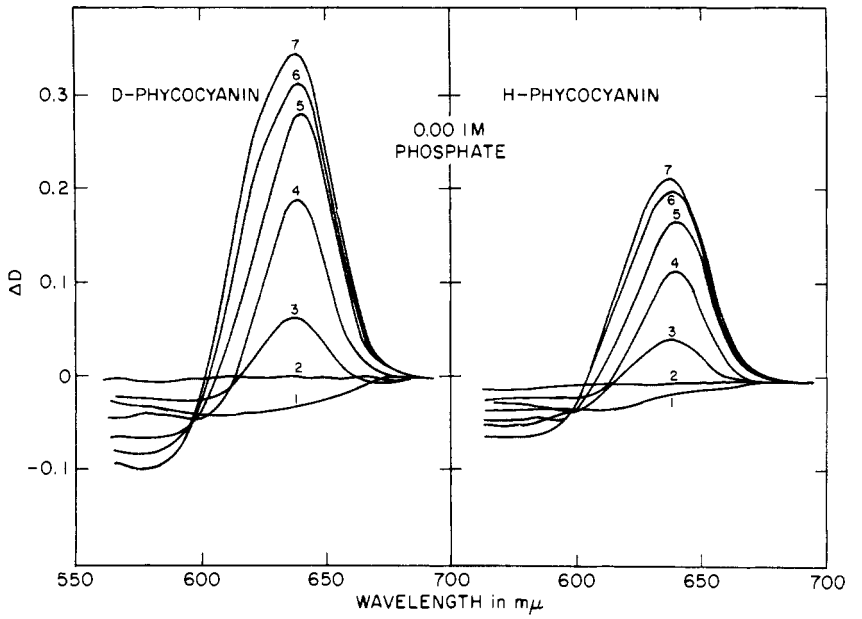

FIGURE 6: Effect of *p*H on the difference spectra of phycocyanin in 0.001 M phosphate at very low protein concentration. Left, deuteriophycocyanin, 0.36 mg/ml; right, protiophycocyanin, 0.23 mg/ml. Curve 1, *p*H 7.4; curve 2, *p*H 7.3; curve 3, *p*H 7.0; curve 4, *p*H 6.5; curve 5, *p*H 5.9; curve 6, *p*H 5.7; curve 7, *p*H 5.5. Reference: *p*H 7.3.

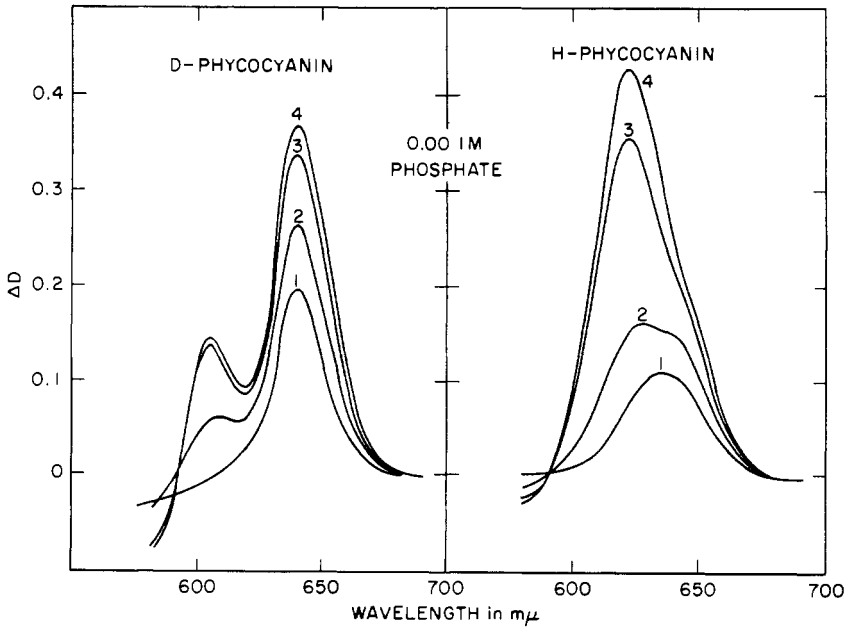

FIGURE 7: Effect of *p*H on the difference spectra of phycocyanin in 0.001 M phosphate at moderate protein concentration. Left, deuteriophycocyanin, 2.3 mg/ml; right, protiophycocyanin, 1.5 mg/ml. Curve 1, *p*H 6.5; curve 2, *p*H 6.2; curve 3, *p*H 5.8; curve 4, *p*H 5.6. Reference: *p*H 6.8.

within the limit of experimental error, with the values reported in the literature (Svedberg and Katsurai, 1929; Svedberg, 1937; Hattori and Fujita, 1959).

On the other hand, we can estimate the molecular weight of deuteriophycocyanin from the hydrogen content, fraction of nonexchangeable hydrogen, and molecular weight of protiophycocyanin. The fraction of nonexchangeable hydrogen was estimated to be 75% from the amino acid composition data (Berns *et al.*, 1963). The molecular weights of deuteriophycocyanin thus calculated are 141,000 in the S-6 form and 278,000 in the S-11 form, which coincide nicely with values calculated from sedimentation and diffusion data.

A lower molecular weight unit may be observed

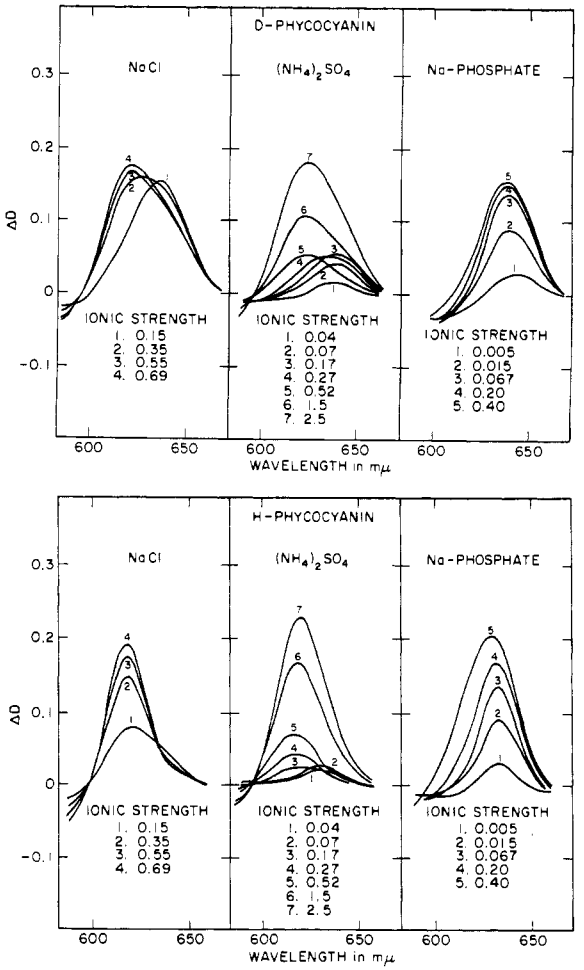

FIGURE 8: The effect of ionic strength on the difference spectra of phycocyanin at *p*H 7.0. Top: deuteriophycocyanin; left, 0.19 mg/ml; middle, 0.25 mg/ml; right, 0.18 mg/ml. Bottom: protiophycocyanin; left, 0.19 mg/ml; middle, 0.19 mg/ml; right, 0.23 mg/ml. References: left, 0.01 M phosphate, $\mu = 0.02$; middle, 0.01 M phosphate, $\mu = 0.02$; right, 0.001 M phosphate, $\mu = 0.0016$.

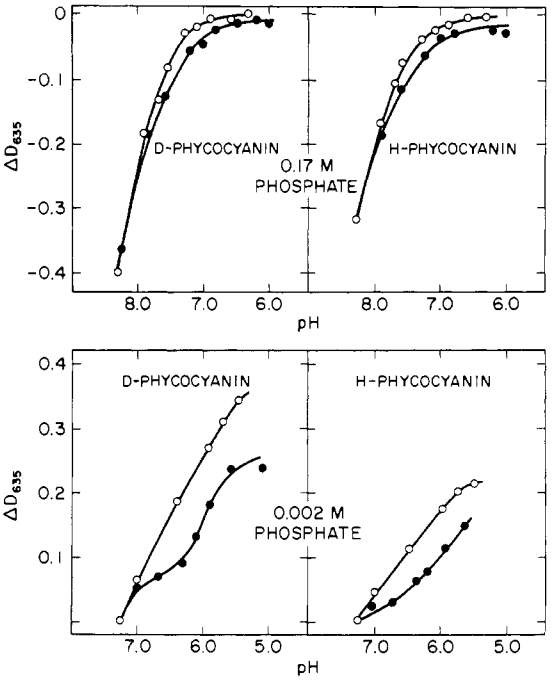

FIGURE 9: Reversibility of *p*H-induced changes in phycocyanin. Absorption changes were determined at 635 mμ. Top: 0.17 M phosphate; open circles, *p*H shifted from acidic to alkaline; closed circles, *p*H shifted from alkaline to acidic. Concentration of deuteriophycocyanin, 0.24 mg/ml; of protiophycocyanin, 0.24 mg/ml. Bottom: 0.002 M phosphate; open circles, *p*H shifted from acidic to neutral; closed circles, *p*H shifted from neutral to acidic. Concentration of deuteriophycocyanin, 0.40 mg/ml; of protiophycocyanin, 0.23 mg/ml.

TABLE VI: Molecular Weight of Phycocyanin.

Phycocyanin	*p*H 7.0	*p*H 5.3
Protiophycocyanin[a]	134,000	266,000
Deuteriophycocyanin[a]	138,000	275,000
Deuteriophycocyanin[b]	141,000	278,000

[a] Molecular weight calculated from sedimentation and diffusion. [b] Molecular weight calculated from hydrogen content, fraction of nonexchangeable hydrogen, and molecular weight of protiophycocyanin.

(Tables I and II) with a sedimentation coefficient of about 3. No unequivocal value for the diffusion constant of this component is available. Judging from the sedimentation values we postulate (*vide infra*) that three S-3 components associate to form one S-6 component. Thus we can assign 46,000 as the molecular weight of the S-3 component. The minimum molecular weight of phycocyanin has been calculated to be 15,200 from its amino acid composition (Berns *et al.*, 1963), which supports our assumption.

More recently, Berns *et al.* (1964) have published some phycocyanin diffusion coefficients obtained by an immunological technique. This measurement was conducted at *p*H 7.0, so that the major component is most likely the S-6 component. A diffusion coefficient of 4.2 $\times 10^{-7}$ cm²/sec was measured for the main precipitation line, a value in good agreement with the data reported here. Berns *et al.* (1964) also found two faster-diffusing species, 7.45 $\times 10^{-7}$ cm²/sec and 1.32 $\times 10^{-6}$ cm²/sec, which he assigned to S-6 and S-3, respectively. We believe the diffusion coefficient 7.45 $\times 10^{-7}$ cm²/sec belongs to the S-3 component, and in our opinion the molecular weights calculated by Berns (95,000 for S-6 and 28,000 for S-3) are too low. If we combine the intermediate diffusion coefficient, 7.45 $\times 10^{-7}$ cm²/sec, with the sedimentation coefficient, 3.76 $\times 10^{-13}$ sec^{-1}, we calculate the molecular weight of S-3 to be 48,100, a value in good agreement with that calculated from our

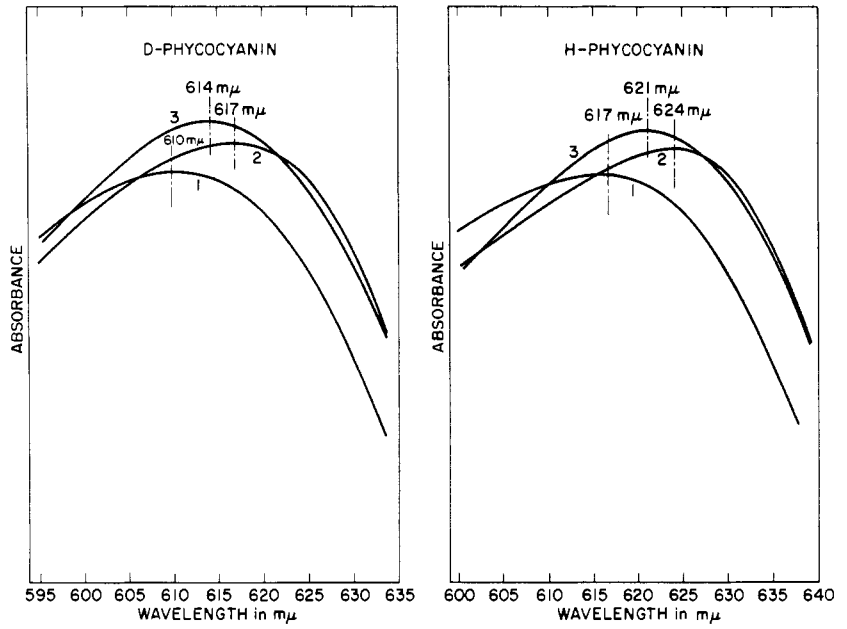

FIGURE 10: Absorption spectra of phycocyanin in the red band region. Left: deuteriophycocyanin. Curve 1, *p*H 7.0, 0.001 M phosphate, concentration of phycocyanin 0.013 mg/ml; curve 2, *p*H 7.0, 0.01 M phosphate, 6.4 mg/ml; curve 3, *p*H 5.3, 0.01 M phosphate, 7.7 mg/ml. Right: protiophycocyanin. Curve 1, *p*H 7.0, 0.001 M phosphate, 0.015 mg/ml; curve 2, *p*H 7.0, 0.01 M phosphate, 1.9 mg/ml; curve 3, *p*H 5.3, 0.01 M phosphate, 6.8 mg/ml.

trimer hypothesis, 46,000. However, an explanation for the fastest-diffusing component of Berns *et al.* (1964) is still lacking.

Absorption Spectra. Marked changes in the absorption spectrum of phycocyanin occur in the region between 600 and 650 mμ (Figure 4), and consequently difference spectra in this region were examined intensively. Figure 5 shows typical examples of the effect of *p*H on absorption spectra of deuterio- and protiophycocyanin at protein concentrations of 0.2–0.3 mg/ml in 0.01 M phosphate buffer. Lowering the *p*H from 7.3 results first in an increase in absorption at about 635 mμ, followed by an increase at about 620 mμ. The second step of the spectral change was more marked with protiophycocyanin. With deuteriophycocyanin, the shorter-wavelength sides of the difference spectra were enhanced to some extent at lower *p*H, but no peak was observed at 620 mμ.

If the concentration of the buffer was reduced to 0.001 M, the second step of the change was suppressed almost completely (Figure 6). When a similar experiment in 0.001 M phosphate was performed at much higher concentrations of phycocyanin (1.5–2.3 mg/ml), absorption changes were observed at 620 mμ, preceded by changes at about 635 mμ (Figure 7).

The effects of variations in ionic strength at neutral *p*H are illustrated in Figure 8. The spectral changes observed were essentially the same as those induced by the lowering of *p*H, that is, an increase in ionic strength is analogous to an increase in hydrogen ion concentration. However, the effectiveness of different salts in this regard was different.

The changes in absorption spectra are reversible within certain limits of *p*H and ionic strengths. Below *p*H 5 and above 7, the spectral changes are to a considerable extent irreversible, as is also the case at lower ionic strengths. In 0.002 M phosphate buffer, a change of *p*H from 5.5 to 7.2 and then back to the original *p*H resulted in the denaturation of 40% of the protein, whereas in 0.17 M phosphate buffer the protein is almost completely recovered even after exposure to much higher *p*H (Figure 9).[1]

Absorption Spectra. Figure 10 shows the absorption spectra of deuterio- and protiophycocyanin under three specified conditions under which phycocyanin is expected to exist, as, respectively, S-3, S-6, or S-11. Shifts at the red end of the visible region are obviously dependent on the association state of phycocyanin. Although it is rather difficult to determine the exact positions of absorption peaks on account of the broadness of their absorption bands, individual peaks are centered approximately at 617, 624, and 621 mμ for protiophycocyanin, and at 610, 617, and 614 mμ for deuteriophycocyanin. The same observations were made with phycocyanin obtained from another strain of blue-green alga, *Phormidium luridum* (DaBoll *et al.*, 1962). Thus the possibility may be excluded that association is a characteristic only of *Plectonema* phycocyanin. Our attempts to resolve the spectra of mixtures of S-3 and S-6 and of S-6

[1] These data bear directly upon the procedures used in the preparation of phycocyanin. Phycocyanin should be kept within the *p*H region 5–7 during extraction and purification.

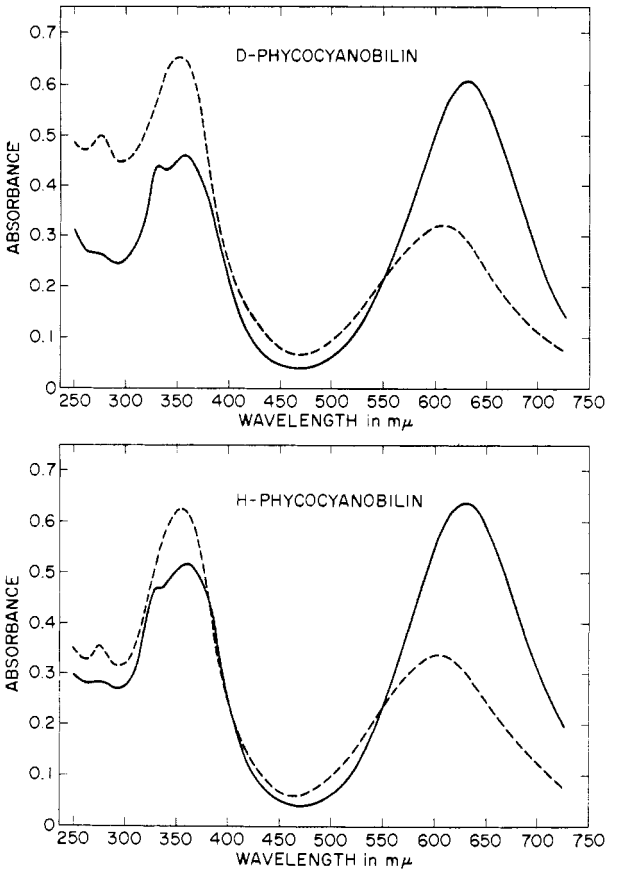

FIGURE 11: Absorption spectra of phycocyanobilin prepared by Ó hEocha's (1958, 1963) HCl method. Top, phycocyanobilin obtained from deuteriophycocyanin in chloroform (dashed line) and in HCl-chloroform (solid line). Bottom, phycocyanobilin obtained from protiophycocyanin in chloroform (dashed line) and in HCl-chloroform (solid line). Examination of the red band on an expanded scale showed little or no shift due to deuterium substitution.

and S-11 by measurements at liquid nitrogen temperature were unsuccessful.[2]

For all three association states the deuteriophycocyanin peaks are located at wavelengths about 7 mμ shorter than those of protiophycocyanin. A blue shift of absorption effected by deuterium substitution has previously been reported for chlorophyll *a* and *b* (Strain *et al.*, 1963), α- and β-carotene, and lutein (Strain *et al.*, 1961). The deuterium shift of the phycocyanin absorption peaks is quite large compared to those observed for the chlorophylls (*ca.* 1 mμ) and carotenoid pigments (2–3 mμ). On the other hand, the absorption spectra of the phycocyanobilin (chromophore group) of protio- and deuteriophycocyanin showed absorption peaks at almost the same wavelengths (Figure 11). Thus, it is likely that deuteration of the protein moiety of phycocyanin is the primary cause of the large spectral shift, presumably through an interaction between the protein and the chromophore groups. This conclusion is supported by the experimental observation that changes in association state of the protein result in a shift of absorption peaks.

Association-Dissociation Equilibrium. The observed spectral changes and the sedimentation behavior can be interpreted either in terms of a molecular association-dissociation involving two consecutive reactions, $6\,P \rightleftarrows 2P_3 \rightleftarrows P_6$, or two parallel reactions, $3\,P \rightleftarrows P_3$ and $6\,P \rightleftarrows P_6$, where P refers to monomeric phycocyanin. At low concentration of phycocyanin, low ionic strength, and neutral *p*H, phycocyanin exists predominantly in the form of monomeric P. An increase in the concentration of phycocyanin or in the ionic strength, or a lowering of *p*H is favorable to the transformation $P \rightarrow P_3$. Similarly, a further lowering of *p*H or an increasing ionic strength or protein concentration favors the formation of P_6. Deuteriophycocyanin is associated to a lesser extent under all conditions studied.

Sedimentation data imply that at high protein concentration and low *p*H phycocyanin exists in a fully aggregated state (molecular weight 270,000) and at high *p*H in a less aggregated state (molecular weight 140,000). At very low phycocyanin concentrations, one

[2] The data presented in Figure 10 may explain the discrepancies among the peak wavelength values reported in the literature (625–615 mμ) (Haxo *et al.*, 1955); the exceptionally low value of 612 mμ reported by Haxo *et al.* (1955) for *Phormidium fragile* phycocyanin may be due to irreversible denaturation.

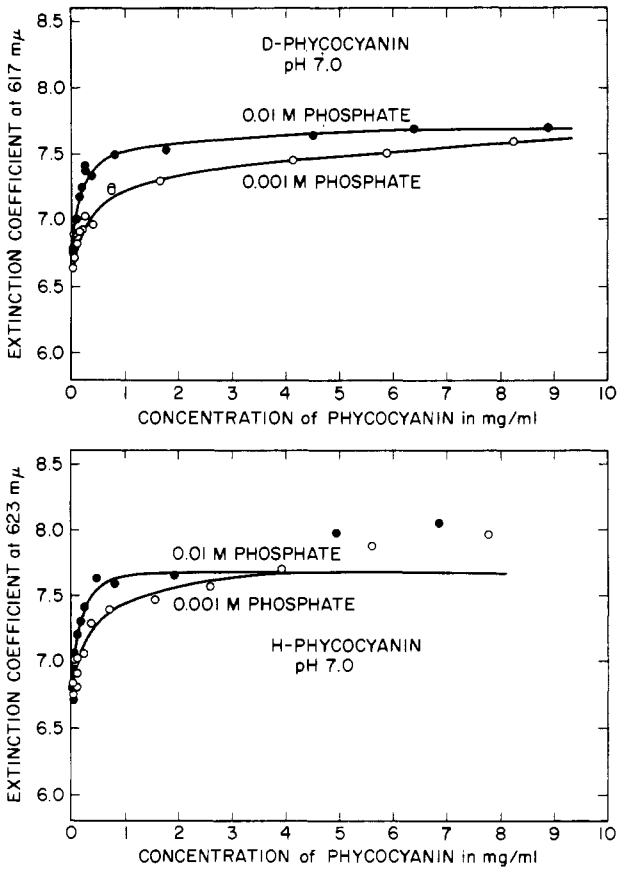

FIGURE 12: Concentration dependency of the extinction coefficient, $E_{1cm}^{mg/ml}$, of phycocyanin at *p*H 7.0. Top, deuteriophycocyanin; bottom, protiophycocyanin.

may observe a monomer unit with an assigned molecular weight of 46,000. Qualitatively, then, the spectral and molecular weight data are compatible with a monomer-trimer-hexamer hypothesis.

To determine equilibrium constants, the extinction coefficients of deuterio- and protiophycocyanin were determined as a function of protein concentration. These experiments were carried out under the following conditions: (1) *p*H 7.0 and 5.3, 0.001 M phosphate; (2) *p*H 7.0 and 5.3, 0.01 M phosphate. The optical absorptivity (Figures 12 and 13), expressed on a dry weight basis ($E_{1cm}^{mg/ml}$), increases with increasing protein concentration. At the very lowest concentrations, a semilog plot makes possible extrapolation to zero concentration, and an absorptivity of 6.4 ± 0.1 was obtained at both *p*H 5.3 and 7.0 for protio- and deuteriophycocyanin. At *p*H 7.0 deuteriophycocyanin in 0.01 M phosphate (Figure 12, top) gives a limiting value for the absorptivity of 7.65 for the S-6 component. The curve for protiophycocyanin shows deviations because of further association at higher protein concentrations, as indicated by difference spectra and sedimentation data. At *p*H 5.3 the best value for the extinction coefficient of the S-11 component is derived from the data for protiophycocyanin in 0.01 M phosphate buffer (Figure 13, bottom), and the absorptivity of the S-11 component is taken to be 8.9. At *p*H 5.3 deuteriophycocyanin is less associated into the S-11 form so that the curves in Figure 13, top, are obtained. We are assuming here, of course, that any differences in extinction coefficient between protio- and deuteriophycocyanin are not large.

The equilibrium constant, K_3, for an association 3 P $\rightleftarrows$ P_3 is given by

$$\frac{c^2\Phi^3}{1-\Phi} = K_3 \qquad (1)$$

where Φ is the weight fraction of P, and c is the concentration of phycocyanin in mg/ml. The values of Φ can be estimated from the data on the concentration dependence of the extinction coefficient of phycocyanin at *p*H 7.0. From sets of values for $\Phi_{1/2}$ and $c_{1/2}$ we can calculate K_3 (Table VII), and this calculated value can in turn be used to determine the fit of the data over the entire concentration range (Figure 14). The good fit supports the use of equation (1) to describe the association of phycocyanin at *p*H 7.0. In terms of K_{3D}/K_{3H}, deuteriophycocyanin exhibits an association tendency about one-fourth that of ordinary phycocyanin.

The equilibrium constant K_3 holds only at *p*H 7.0. Neglecting trimers, the equilibrium constant for associa-

TABLE VII: Dissociation Constants of Deuterio- and Protiophycocyanin.

	Solvent			
	0.001 M Phosphate		0.01 M Phosphate	
	*p*H 7.0	*p*H 5.3	*p*H 7.0	*p*H 5.3
K_{3D}	4.0×10^{-2}		1.5×10^{-2}	
K_{3H}	1.0×10^{-2}		0.4×10^{-2}	
K_{3D}/K_{3H}	4.0		3.8	
$\sqrt{K_{3D}/K_{3H}}$	2.0		2.0	
K_{6D}		2.4×10^{-3}		1.4×10^{-4}
K_{6H}		5.9×10^{-6}		1.0×10^{-7}
K_{6D}/K_{6H}		4.1×10^{2}		1.4×10^{3}
$\sqrt[5]{K_{6D}/K_{6H}}$		3.3		4.3

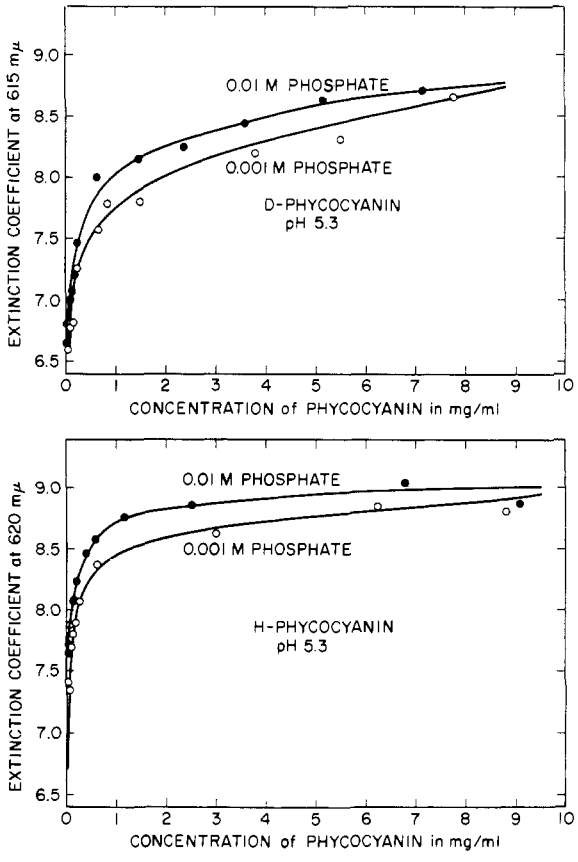

FIGURE 13: Concentration dependency of the extinction coefficient, $E_{1cm}^{mg/ml}$ of phycocyanin at *p*H 5.3. Top, deuteriophycocyanin; bottom, protiophycocyanin.

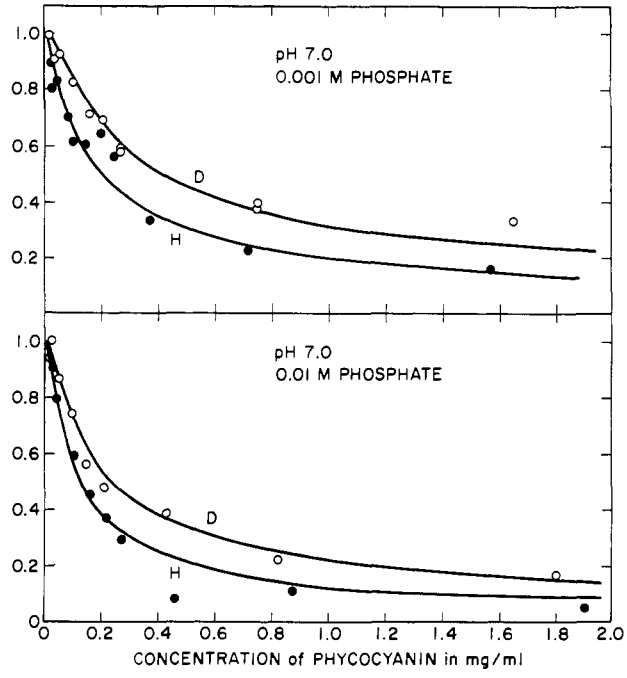

FIGURE 14: Association-dissociation equilibrium of phycocyanin at *p*H 7.0. The weight fractions of monomer, ϕ, were obtained from the data of Figure 12. Curves are calculated from equation (1). Top, 0.001 M phosphate, $K_{3D} = 4.0 \times 10^{-2}$, $K_{3H} = 1.0 \times 10^{-2}$; bottom, 0.01 M phosphate, $K_{3D} = 1.5 \times 10^{-2}$, $K_{3H} = 0.4 \times 10^{-2}$. Left-hand ordinate scaled in units of Φ.

tion to P_6 at *p*H 5.3 was calculated from the equation

$$\frac{c^5\Phi^6}{1-\Phi} = K_6 \qquad (2)$$

where K_6 represents the dissociation constant for the reaction $6\ P \rightleftarrows P_6$.

Figure 15 gives the relation between Φ and c, where curves are drawn using K_6 values from Table VII. A fairly good fit is obtained, indicating that the equilibrium at *p*H 5.3 is represented reasonably well by equation (2). Here, too, deuteration reduces the association tendency. In Table VII, the ratios of K_D/K_H are also given in the form of square root and fifth root to emphasize the difference in interactions between each monomer.

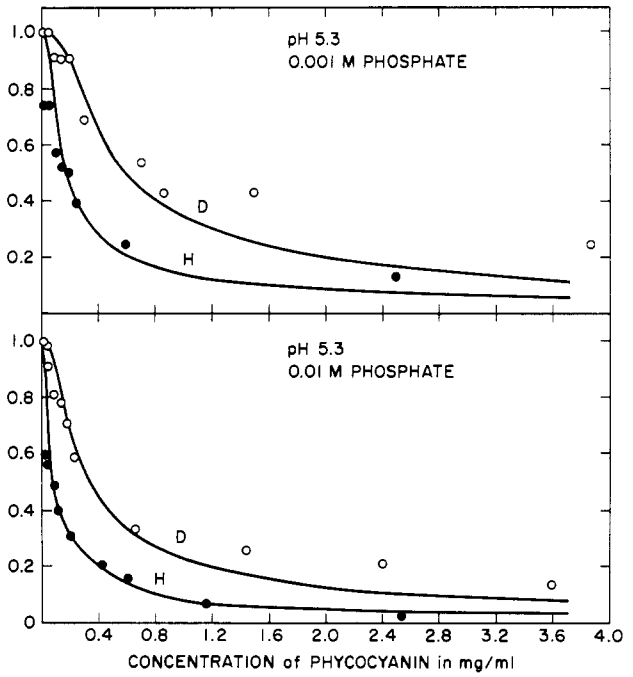

FIGURE 15: Association-dissociation equilibrium of phycocyanin at *p*H 5.3. The weight fractions of monomer, ϕ, were obtained from the data of Figure 13. Curves are calculated from equation (2). Top, 0.001 M phosphate, $K_{6D} = 2.4 \times 10^{-3}$, $K_{6H} = 5.9 \times 10^{-6}$; bottom, 0.01 M phosphate, $K_{6D} = 1.4 \times 10^{-4}$, $K_{6H} = 1.0 \times 10^{-7}$. Left-hand ordinate scaled in units of Φ.

Discussion

Many types of bondings have been invoked to account for the association-dissociation of proteins (Reithel, 1963). Since the isoelectric point of phycocyanin is located between 4.3 and 4.7 (Hattori and Fujita, 1959), phycocyanin molecules are negatively charged in the *p*H region where reversible association-dissociation occurs. Increase in the hydrogen ion concentration thus results in a reduction of net charge, that is, a reduction of electrostatic repulsive forces. The attractive forces remain unchanged. This leads to the association of the monomers. An increase in ionic strength might exert a similar effect by reduction of electrostatic interactions. Side-chain deuteration will change the ionization constants of acidic and basic groups only slightly (Ropp, 1960; Bell and Jensen, 1960; Halevi, 1960; Halevi *et al.*, 1963; Streitwieser and Klein, 1963; Bell and Miller, 1963; Bernasconi *et al.*, 1963), and this change would result in a small shift to higher *p*H of the isoelectric point. If the association of monomers is simply regulated by electrostatic forces, the association tendency of deuteriophycocyanin would be expected, contrary to observation, to be higher than that of protiophycocyanin.

Previous communications (Berns *et al.*, 1963; Hattori *et al.*, 1965) have shown that side-chain deuteration of phycocyanin appears to lessen the side-chain interactions. The present results receive a reasonable explanation by postulating that hydrophobic side chains participate in an important and specific way in the union of subunits, and that deuteration reduces the extent of interaction between hydrophobic side chains of individual monomers. The possible participation of hydrophobic side chains in protein association reactions has been suggested by Kauzmann (1959) and Scheraga (1961), and has been taken into account by Steinberg and Scheraga (1963) in their recent statistical mechanical calculation of the entropy of protein dimerization.

Acknowledgment

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Optical Activity and the Conformation of Polyinosinic Acid and Several Other Polynucleotide Complexes*

P. K. Sarkar and Jen Tsi Yang

ABSTRACT: The optical rotatory dispersion (ORD) and absorption spectra of poly-I, poly-C, poly-(I + C), poly-(A + 2I), and poly-(G + C) were measured at various temperatures. All the polymers exhibited multiple Cotton effects below 300 mμ, but poly-I is the first polynucleotide studied that shows two troughs and one peak between 240 and 300 mμ instead of two peaks and one trough in the same wavelength range, as observed for nucleic acids and other polynucleotides. The complex formations of poly-I with poly-C or with poly-A immediately inverted the ORD profile to one characteristic of other polynucleotides.

This must be attributed to the difference in base interactions, since an inverse ORD profile for polynucleotides does not necessarily indicate a change in the handedness of the helices. In all cases the temperature curves of ORD paralleled those of the hyperchromic effect of the polymers. Poly-I showed a broad melting temperature in 0.01 M NaCl but a sharp transition in 1 M NaCl. This supports the contention that poly-I in low salt solution has very little, although not negligible, secondary structure. The three complexes, poly-(I + C), poly-(A + 2I), and poly-(G + C), all showed sharp helix-coil transitions, some of which were irreversible and some partially reversible.

ORD[1] of nucleic acids and polynucleotides in the visible region shows that their secondary structure generally contributes a positive rotation to that of their constituent mononucleotides (Doty *et al.*, 1959; Ts'o *et al.*, 1962; Samejima and Yang, 1964, 1965; Sarkar and Yang, 1965a). All these polynucleotides exhibit multiple Cotton effects with two peaks and one trough in the wavelength range of 230 and 300 mμ, which are drastically diminished at elevated temperature (Samejima and Yang, 1964, 1965; Sarkar and Yang, 1965a). In this communication we report the ORD of poly-I, since inosine is an analog of guanosine while poly-G is difficult to prepare and not available to us (Fresco and Su, 1962). We will show that poly-I is the first polynucleotide studied that distinguishes itself from the others by having two troughs and one peak between 240 and 300 mμ. Once poly-I is complexed with poly-C or poly-A, however, the ORD profiles immediately reverse to those resembling nucleic acids

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[1] Abbreviations used in this work: ORD, optical rotatory dispersion; IMP, inosine 5′-phosphate; AMP, adenosine 5′-phosphate; CMP, cytidine 5′-phosphate; T_m, melting temperature.