Protein–Protein Interaction. The Phycocyanin System*

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ABSTRACT: The C-phycocyanin system has been studied by sedimentation velocity as a function of pH, ionic strength, temperature, and buffer. Studies of the pH dependence of the viscosity and absorption spectrum are also presented. The data are interpreted in terms of a postulated monomer, trimer, hexamer, and dodecamer equilibrium. The trimer and hexamer are the predominant species under the conditions investigated. The thermodynamic trends associated with the equilibria are discussed in terms of the probable forces involved in the specific interactions. Analysis of diffusion coefficient for the hexamer, as determined from immunodiffusion and the intrinsic viscosity, led to axial ratios characteristic of a prolate and oblate ellipsoid which are in substantial agreement with a model for the hexamer proposed on the basis of electron micrographs of the protein.

The presence of the hexamer structure is demonstrated to be consistent with reported fluorescence depolarization studies. Chlorophyll–phycocyanin hexamer complexes are suggested for optimal energy transfer in blue-green algae.

Phycocyanin, a chromoprotein from blue-green algae, has been investigated in some detail (Ó hEocha, 1962). These studies have not included a substantial physical–chemical series of experiments that unambiguously characterize the physical nature of the system, although several physical–chemical investigations are reported.[1] Interest in attempting a definitive characterization of this system arises both from our past (Berns, 1963a,b) and current work on fully deuterated phycocyanin. The fully deuterated protein offers an exceptional opportunity for elucidation of several forces involved in the stabilization of secondary, tertiary, and quaternary protein structure. Utilization of this potential requires an extremely well-characterized normal system. Our purpose is to contribute information that will present a characterization of the normal phycocyanin system, which itself is a tool for investigating the forces involved in quaternary protein structure.

Experimental

Materials and Methods. All studies, unless otherwise stated, were performed with phycocyanin obtained from the alga *Plectonema calothricoides*. Phycocyanin from the alga *Phormidium luridum* was used when specifically noted. Cultures of both algae were obtained from the Indiana University culture collection. The protein was usually extracted by lysing cells with lysozyme. Cell debris was removed by dissolving the sample in pH 7.0 sodium phosphate buffer and by centrifuging for 10 min at 12,000 rpm in a Servall RC-2 refrigerated centrifuge. The protein was purified by the ammonium sulfate procedure (Berns *et al.*, 1963) until a ratio of the 620-mμ/280-mμ absorption was greater than 4.0. An additional step in this procedure consisted of repeated precipitation with 35% saturated ammonium sulfate. The supernatant was found to contain allophycocyanin precipitated when 50% saturated ammonium sulfate was used. To assure that the protein isolated was not uniquely dependent upon the procedure, additional

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[1] After submission of this manuscript for publication, the paper of Hattori *et al.* (1965) appeared. Although this work represents an interesting contribution, it is not pertinent to the nature of the native phycocyanin system for several reasons. Use of the calcium phosphate or hydroxylapatite procedure or the ion-exchange celluloses for purification of this protein is demonstrated to cause an irreversible change in the aggregation of this system. The characterization of the calcium phosphate purification procedure is considered in detail in this publication. Essentially the same observations are true for the cellulose ion-exchange procedures. The behavior observed by Hattori *et al.* (1965) is analogous in most respects to that observed in our samples when treated by any of these chromatographic procedures. It is also important to realize that very high concentrations of this protein should be examined to make relevant comments about the *in vivo* nature of the protein, particularly since the protein is found to represent as much as 25% of the total weight of a blue-green alga. The highest concentration examined in the study of Hattori *et al.* (1965) is about 10 mg/ml. In addition, only two pH values are examined and no temperature dependence of sedimentation. These facts make it difficult to comment in a relevant fashion on the significance of their data. Our general observations on the nature of the aggregation phenomena reported in the present publication have been corroborated in this laboratory by similar studies, to be reported shortly, on other phycocyanins including a fully deuterated phycocyanin and one from a thermophilic alga.

experiments were performed with protein extracted from algae without prior freezing, by lysozyme treatment, by freezing and thawing, or by macerating with sand. All these gave extracts exhibiting very little apparent variation. Purification by column chromatography of the phycocyanin was also investigated. The columns[2] used included Sephadex G-50, G-75, G-100, and G-200 and Sephadex A-50-DEAE, as well as Mannex ECTEOLA and DEAE-celluloses. Results with these columns were successful to varying degrees; however, with the OD ratio of 620 mμ/280 mμ as a criterion no procedure was more successful than the simple ammonium sulfate fractionation. Chromatography with hydroxylapatite and calcium phosphate gel, according to the procedures of Haxo *et al.* (1955) and Tiselius *et al.* (1956), modified the protein irreversibly, as determined from an ultracentrifugation in which the number and sedimentation rate of the peaks present were determined as a function of pH. Similar effects were observed with preparations with the ion-exchange celluloses.

The purified protein was stored at ~4° under 50% saturated ammonium sulfate until use. Protein solutions were allowed to dialyze overnight against the appropriate buffers with at least two changes of buffer. Buffers were prepared to $\mu = 0.1$, unless otherwise stated. The pH was checked with a Radiometer TTT1C pH meter with the pH adjusted to ±0.05. All protein concentrations were determined by the Kjeldahl method as modified by Markham for micro amounts reported in Kabat and Mayer (1961). Kjeldahl determinations were performed on the dialysates to determine that residual ammonium sulfate was not present in sufficient quantity to cause errors in the protein determination. The per cent nitrogen in the *P. calothricoides* phycocyanin was determined previously (Berns *et al.*, 1963).

Ultracentrifuge experiments were performed on a Spinco Model E ultracentrifuge, with a Philpot-Svenson cylindrical-lens diagonal-bar system. Type 1N Kodak spectroscopic plates and a Corning No. 5031 filter were used because of the intense blue absorption of the protein.[3] All runs were done at 25 and 60° bar angle, except when otherwise noted. Studies were performed at several concentrations at each pH investigated. The sedimentation coefficients were extrapolated to zero concentration. In the temperature–sedimentation studies dialysis overnight at the temperature of the centrifugation was possible when low temperatures were investigated. At higher temperatures this was not practical because of the relative lability of the protein. Equilibration at the centrifugation temperature for 1 hr or longer gave reproducible results. Therefore, it was assumed that equilibrium was achieved at these higher temperatures within 1 hr and quite likely within minutes. All distances were measured on a Nikon microcomparator. Relative areas were measured from enlarged tracings of the centrifuge patterns by a planimeter or by counting squares on appropriate graph paper. Corrections for radial dilution were investigated; however, these corrections were not large enough compared with the error in calculation of the relative areas to warrant consideration.

Diffusion studies were performed by the immunodiffusion method of Allison and Humphrey (1960), and attempts were made to use the sedimentation boundary spreading method of Fujita as modified by Baldwin (1957) for the sedimentation studies. The free-solution diffusion method was attempted on the Spinco Model H electrophoresis diffusion apparatus. The immunodiffusion studies were carried out with phycocyanin antigen prepared by ammonium sulfate and by the calcium phosphate gel procedure. Rabbit antibody was prepared as previously reported (Berns, 1963b). Viscosity measurements were carried out with a Cannon-Ubbelohde semimicroviscometer, size 25. This instrument was calibrated with dioxane, with the National Bureau of Standards calibrated viscometer as a reference. All measurements were at 25.00 ± 0.02° and times were recorded to tenths of a second with an electric timer. Solutions were filtered before use through a coarse fritted disk under their own hydrostatic head. The instrument was cleaned after each protein dilution. The method of least squares was used to evaluate the data. The extrapolation to infinite dilution gave the kinematic viscosity accurate to ±5%. The results were not corrected to the true intrinsic viscosities (Tanford, 1955) since the correction in this case would be less than the experimental error. To investigate further the reliability of the viscometers, the intrinsic viscosity of ovalbumin was determined. An $[\eta]$ of 4.2 cc/g was calculated as compared to a value of 4.3 reported by Polson (1939).

The apparent partial specific volume of the phycocyanin was determined by the pycnometric method with a Lipkin 1-ml pycnometer. The pycnometer was calibrated with distilled water. All determinations were made at 25.00 ± 0.02°. The precision of the density measurements was ±0.0003 g/ml. The apparent partial specific volume was calculated according to the equation

$$\phi = \frac{1}{P_s}\left(1 - \frac{\Delta P}{C}\right)$$

where ϕ is the apparent partial specific volume, P_s is the buffer solution density, and ΔP is the difference between the density of the solution of concentration C and the buffer solution density. The true partial specific volume was not calculated, since the error in the apparent partial specific volume is larger than the difference between the apparent and true partial specific volume. In the pH range 5.0–9.0 the average partial specific volume was 0.75 ± 0.01. From the known amino acid analysis (Berns *et al.*, 1963) and the method of Cohn and Edsall (1943) a partial specific volume of

[2] The Sephadex G-100 system has been investigated in some detail and there is a definite indication of separations in this system, according to molecular size. At present we have not achieved complete separation of any one species by this method.

[3] At 622 mμ the protein has an optical density of 6.0. for a 0.1% solution in a 1-cm cell.

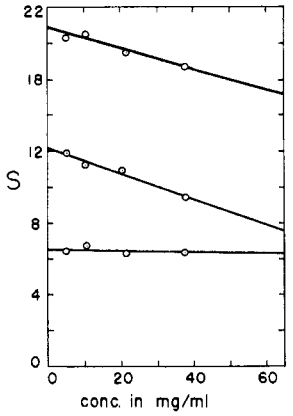

FIGURE 1: A plot of sedimentation coefficient as a function of concentration for the observed species in phycocyanin from *P. calothricoides* at pH 5.0, acetate, μ = 0.1, and 25°.

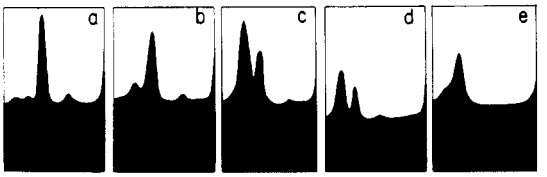

FIGURE 2: Typical sedimentation studies of phycocyanin from *P. calothricoides*. All sedimentation is from left to right at 59,780 rpm; each picture is at 40 min, 25°, and ionic strength of 0.1 at each pH: (a) pH 5.0, acetate; (b) pH 6.0, phosphate; (c) pH 7.0, phosphate; (d) pH 8.0, phosphate; (e) pH 9.0, carbonate.

0.753 was calculated. The buffers used are: pH 5.0, μ = 0.1, acetate; pH 6.0, μ = 0.1, phosphate; pH 7.0, μ = 0.1, phosphate; pH 7.0, adjusted phosphate buffer 2.5 M in sodium chloride; pH 7.0, μ = 0.025, phosphate; pH 7.0, μ = 0.1, cacodylate; pH 8.0, μ = 0.1, phosphate; pH 9.0, μ = 0.1, carbonate.

Results

Sedimentation. The results of the sedimentation studies with C-phycocyanin from *P. calothricoides* are shown in Table I. The $s_{0,25}$ values listed are a result of extrapolation of s_{25} *vs.* concentration plots (*e.g.*, Figure 1). The relative amounts of each species present at each pH are also given. The per cent of each species found at a specific pH and ionic strength in the protein concentration region investigated was apparently independent of total concentration of protein and time of sedimentation. Several experiments carried out at slower sedimentation speeds also indicated no change in the number of peaks present and essentially the same sedimentation coefficients and distribution of species. In a double-sector cell the schlieren pattern between peaks was observed to return to the base line.

The system exhibited reversible behavior in the pH 5.0–9.0 range as judged from sedimentation patterns. Dialysis and redialysis of aliquots of samples from one pH to another resulted in reproducible centrifuge patterns. The concentration *vs.* sedimentation coefficient plot for all species exhibited a negative slope with no detectable signs of curvature.

The sedimentation behavior is quite sensitive to pH (Table I). The isoelectric point of this protein has been reported by many investigators (Ó hEocha, 1962) to be pH 4.7, and this result has been confirmed by preliminary free-solution electrophoresis experiments in this laboratory. At pH 5.0, μ = 0.1, 7S, 11S, and 19S species are present. At a high enough concentration it is possible to detect a 3.76S peak (Figure 2a). The 11S species is most heavily favored. As the pH is increased at constant ionic strength the 11S species becomes less predominant. At pH 7.0 the 7S species becomes predominant. At pH 9.0 no 11S material is detected. If cacodylic acid buffer of the same ionic strength at pH 7.0 was used in place of phosphate buffer, it was observed that the 11S species became predominant and the relative amount of 19S material increased. This would seem to indicate a specific buffer ion-binding effect by phosphate ion which is important in perturbation of the association equilibria. Similar pH studies of the sedimentation behavior of C-phycocyanin from *P. luridum* were performed by A. Morgenstern of this laboratory. The results were completely analogous to those reported in Table I.

TABLE I: Sedimentation Behavior of C-Phycocyanin as a Function of pH.

pH[a] (Buffer)	$s_{0,25} \times 10^{13}$	Relative Area under Peaks (%)
5.0 (acetate)	3.7[b]	6
	6.3	10
	12.1	75
	18.9	9
6.0 (phosphate)	6.2	25
	12.6	68
	19.2	8
7.0 (phosphate)	7.0	62
	12.9	32
	20.8	7
7.0 (cacodylate)	6.9[b]	27
	10.2[b]	63
	18.6[b]	11
8.0 (phosphate)	6.5	65
	12.5	31
	20.1	5
9.0 (carbonate)	5.2	93
	18.9	7

[a] Ionic strength is 0.1 for all pH values. [b] *s* values from a single determination, not extrapolated values.

TABLE II: Ionic Strength Studies of Sedimentation at 25°.

Buffer	Relative Area under Peaks (%) 7S	11S	19S
pH 7.0, μ = 0.1, phosphate	62	32	7
pH 7.0 phosphate, 2 M NaCl	30	66	5
pH 7.0, μ = 0.025, phosphate	76	22	2

The effect of ionic strength on the sedimentation behavior of phycocyanin is reported in Table II. The general observation from these results is that at high ionic strength the 11S species is favored and at lower ionic strength the 7S species is more predominant. These results have been confirmed in an even greater range of ionic strengths and pH values with phycocyanin from *P. luridum*.

The effect of temperature on the sedimentation patterns of phycocyanin is presented in Table III. In all

TABLE III: Temperature Studies of Sedimentation.

Buffer and Temp (°C)	Relative Area under Peaks (%) 7S	11S	19S
pH 6.0, μ = 0.1, phosphate buffer[a]			
33.8	17	80	3
25	21	75	4
9.9	45	50	6
pH 7.0, μ = 0.1, cacodylic acid buffer[a]			
22	27	63	11
14.8	44	54	2
5.4	54	43	2

[a] All are the same concentration.

cases investigated decreasing the temperature favors the 7S species at the expense of the 11S species. In phosphate buffer the 19S material also appears to be favored at lower temperatures; however, the area of this peak makes this analytical result less dependable. Sedimentation at 10° on a pH 9.0, μ = 0.1, sample demonstrates the presence of only 3S and 7S material; at pH 5.0, μ = 0.1, and 10°, only 11S material and 3S material were present. The 7S and 11S materials were, respectively, the dominant species in each case.

Experiments with sodium dodecyl sulfate indicate that with increasing detergent concentration slower sedimenting species are favored. Urea experiments demonstrate analogous behavior and redialysis shows that the dissociation perturbation is 75% reversible. Guanidine salts have been used in analogous experiments with *P. luridum* phycocyanin with similar results except that the guanidine salts are more effective perturbants.

Viscosity Studies. The intrinsic viscosity of *P. calothricoides* phycocyanin at several pH values is listed in Table IV. It goes through an apparent maximum in the pH 6.0 region.

TABLE IV: Intrinsic Viscosity (Kinematic) as a Function of pH.

pH[a] (Buffer)	$[\eta]$ (cc/g)
5.0 (acetate)	8.3 ± 0.3
6.0 (phosphate)	9.3 ± 0.6
7.0 (phosphate)	8.4 ± 0.6
8.0 (phosphate)	6.1 ± 0.4
9.0 (carbonate)	3.2 ± 0.2

[a] Ionic strength is 0.1 for all pH values.

Spectra. The visible and ultraviolet absorption spectrum of *P. calothricoides* phycocyanin has been investigated as a function of pH and ionic strength. In Table V are the results for the ratios of the optical densi-

TABLE V: Absorption Spectra Experiments.

pH (Buffer)	622-mμ OD/ 278-mμ OD	622-mμ OD/ 350-mμ OD	278-mμ OD/ 350-mμ OD
5.0 (acetate)[a]	3.0	5.0	1.7
6.0 (phosphate)[a]	3.7	7.0	1.8
7.0 (phosphate)[a]	3.2	5.2	1.6
8.0 (phosphate)[a]	3.0	4.3	1.5
5.0 (acetate)[b]	3.6	6.3	1.7
6.0 (phosphate)[b]	4.2	7.0	1.7
7.0 (phosphate)[b]	3.9	6.8	1.7
8.0 (phosphate)[b]	3.2	5.0	1.6

[a] Ionic strength is 0.1. [b] Buffer is 2 M in NaCl.

ties of the absorption maxima for a typical experiment in which the pH and ionic strength were varied. The 622-mμ/278-mμ OD ratio goes through a maximum at a pH of 6.0, independent of ionic strength. The 622-mμ/350-mμ OD ratio behaves in an analogous fashion. The 278-mμ/350-mμ OD ratio appears to be less sensitive. It seems likely that it is the 622-mμ absorption that is changing. Gel filtration on Sephadex G-100 gives leading fractions with the visible absorption maximum

shifted to 630 mμ and trailing fractions with the maximum shifted to 612 mμ. This long wavelength shift is associated with the heavy molecular weight unit. Analogous shifts in a spectrum as a function of pH are to be expected.

Diffusion Coefficient. Some work was done in free solution diffusion on the Spinco Model H electrophoresis diffusion apparatus. Diffusion coefficients were determined comparable to those reported by Hattori and Fujita (1959) and Tiselius and Gross (1934). However, this procedure was abandoned, since from the sedimentation patterns it was obvious the system is heterogeneous and the heterogeneity is a function of temperature. The immunodiffusion technique of Allison and Humphrey (1960) was used. Phycocyanin purified by ammonium sulfate fractionation was used as the antigen. The multiple lines[4] were successfully analyzed to give the diffusion coefficients listed in Table VI. The experiments were performed at

TABLE VI: Diffusion Coefficients from Immunodiffusion.

$D \times 10^7$ (cm²/sec)
4.2 ± 0.4
7.5 ± 0.6
13.2 ± 1.6

pH 5.0, 6.0, 7.0, 8.0, and 9.0 and the coefficients exhibited little detectable variation with pH. In addition, the experiments were carried out at varying antigen and antibody concentrations to ascertain whether there was any detectable effect of interaction with the media. The number and strength of precipitin lines varied with the concentration, as would be expected. However, the angle the precipitin lines made with the antigen wells was essentially invariant and hence the diffusion coefficients thus determined were reproducible. At least ten determinations for each diffusion coefficient are represented by the result and the standard deviation in the table. Assignment of the diffusion coefficients to the various species was ascertained by using a hydroxylapatite-purified phycocyanin sample. This sample at pH 7.0, $\mu = 0.1$, exhibited a 7S peak and a barely detectable 11S sedimentation peak, and at pH 6.0, $\mu = 0.1$, the 11S peak was the only one present. Immunodiffusion studies at pH 7.0 yielded a single line with a diffusion coefficient of 7.5×10^{-7}. The pH 6.0, $\mu = 0.1$, immunodiffusion study exhibited two lines, with diffusion coefficients of 4.2×10^{-7} and 7.5×10^{-7}. Since at pH 7.0 principally 7S material and only a trace of 11S material were present, the single diffusion coefficient determined at that pH, 7.5×10^{-7}, was assigned to the 7S species. At pH 6.0 in the calcium phosphate gel-treated preparation the presence of an 11S peak in the sedimentation pattern correlated with the appearance of a slower diffusing line (4.2×10^{-7}) in addition to the 7.5×10^{-7} line in immunodiffusion studies made at this pH. The slowest sedimenting species (3S) would be expected to have the largest diffusion coefficient, namely, 13.2×10^{-7}. It was found that the precipitin line representing this diffusion coefficient appeared with increased intensity under conditions favoring the 3S species in sedimentation experiments. Immunodiffusion experiments made in the presence of increasing sodium dodecyl sulfate concentration in the antigen well increased the intensity of the 7.5×10^{-7} and 13.5×10^{-7} lines. At 1% dodecyl sulfate the 13.5×10^{-7} line was either the only line present or the predominant line. Rabbit antibody produced in response to antigen treated with 1% sodium dodecyl sulfate apparently had a great increase in titer directed toward the fastest diffusing antigen. The assignment of the diffusion coefficients was further tested for consistency by comparison with the attempted calculation of diffusion coefficients from the boundary-spreading method of Baldwin (1957) as applied to the sedimentation patterns. The Fujita plots were never quite linear; however, the approximate value arrived at for each S value was in the same range as those determined by immunodiffusion.

Purity Criteria. The procedure for purification of the proteins employed in our studies has avoided column chromatography or any of the calcium phosphate batch-type procedures of other investigators (Haxo *et al.*, 1955; Hattori and Fujita, 1959). Exposure of the protein to calcium phosphate gel or hydroxylapatite is deleterious to the reversible association.

In a typical experiment, phycocyanin purified by ammonium sulfate fractionation was examined on the analytical ultracentrifuge at pH 7.0. The usual 7S, 11S, and 19S species were found and in the expected relative amounts. It should be noted that crude algal extracts examined on the centrifuge also contained these species. The sample was then applied to a calcium phosphate gel column and eluted according to the procedure of Haxo *et al.* (1955). The elution pattern was spectrophotometrically scanned and the blue material (622 mμ), which was over 99% of the sample, was recovered. There was evidence of traces of two small colorless peaks with ultraviolet absorption. The colored material was pooled, precipitated in 50% ammonium sulfate, concentrated, and dialyzed in pH 7.0 and 6.0 buffers. These samples were sedimented. The pH 7.0 sample exhibited one major peak of 7S value with a small amount of 11S material. The pH 6.0 sample exhibited one peak at an 11S value. This is the classical behavior reported by most investigators (*e.g.*, Hattori and Fujita, 1959). Immunodiffusion studies, as recorded in another part of this publication, were consistent with these results. Since the total recovery of protein from the column was over 80% and more than 99% of this was in one fraction, it is a creditable conclusion that there was no fractionation of the major

[4] Note that all precipitin lines fluoresced red when examined under a long wavelength ultraviolet mineral light. This is indicative of the observed phycocyanin fluorescence.

components in the sedimentation pattern of the prechromatographed sample, and the irreversible change found in the chromatographed protein sedimentation pattern is a result of calcium phosphate–protein interaction. This type of purification procedure leads to an apparent irreversible alteration of the system.

Discussion

Macromolecular Aspects. The minimum or monomer molecular weight of C-phycocyanin, as previously reported by Berns *et al.* (1964), is in the 30,000 molecular weight region. A naive application of the Svedberg equation, using the sedimentation coefficients and their respective diffusion coefficients as assigned to them from the immunodiffusion studies, gives a molecular weight for the 3S species and the 7S species of 28,000 and 80,000, respectively. This and minimal molecular weight estimations from these diffusion coefficients and sedimentation coefficients separately lead us to postulate the 7S species to be a trimer of approximately 90,000 molecular weight and the 11S species a hexamer in the 180,000 molecular weight region. The 19S species is assumed to be in the 360,000 molecular weight region and is a dodecamer. In the course of this discussion it will be made clear that the 11S species is quite probably the species of major importance *in vivo*. It is also highly probable that the 19S material is functionally important. At this time we do not have sufficient evidence to speculate intelligently whether the 3S (monomer) and 7S (trimer) are important *in vivo*. The experimental evidence presented here favors the assumption that *in vitro* the following equilibrium exists

$$12M \rightleftharpoons 4M_3 \rightleftharpoons 2M_6 \rightleftharpoons M_{12}$$

where M is the monomer or minimal molecular weight unit. For calculation purposes it is simpler to write the three equations as

$$3M \rightleftharpoons M_3 \qquad K_0 = [M_3]/[M]^3$$
$$2M_3 \rightleftharpoons M_6 \qquad K_1 = [M_6]/[M_3]^2$$
$$2M_6 \rightleftharpoons M_{12} \qquad K_2 = [M_{12}]/[M_6]^2$$

The presentation of the equilibria in the form of monomer, trimer, hexamer, and dodecamer should not be construed to mean that we doubt the existence of intermediate species; however, it is quite likely, judging from the experimental results, that the intermediate species are energetically unfavorable aggregates under the conditions investigated.

In a simple system, the Svedberg equation could be used with the available sedimentation and diffusion data to give the molecular weight of the protein. In this work we have a rather complex situation in which the sedimentation coefficient of each species is affected by the presence of the several interacting species.

There are at least two classes of interactions with which we are concerned. One is the physical interaction of the several particles, which represents the effect on the rate of sedimentation of any of the species due to the presence of the other species. This problem is considered in detail by Schachman (1959) for somewhat simpler systems. The second class of interaction is the association into trimer, hexamer, and dodecamer. The relative rates of association and dissociation for these reactions and their absolute values will determine whether the rate of sedimentation will have an effect on the distribution of the species (Nichol *et al.*, 1964). These two classes of interaction will be considered separately. The first type of interaction is generally treated by a consideration of the Johnston–Ogston effect (Schachman, 1959). In the present work we have at least four interacting species. Therefore, the effect of the interaction on the sedimentation coefficients cannot be simply calculated by the Johnston–Ogston effect. In addition, the observation that the area distribution of species is not a function of total protein concentration under the set of conditions investigated would seem to rule out consideration of the Johnston–Ogston effect.

The sedimentation patterns are such that the species present in any run exhibits the behavior of independent species with no interaction. This is the simplest case of an interacting protein system as described by Nichol *et al.* (1964). The relative area of each species is essentially independent of concentration and time and velocity of sedimentation. The sedimentation coefficients exhibit the simple concentration dependence as shown in Figure 1. It should be pointed out that the data of Figure 1 do not go to sufficiently low concentrations to eliminate the possibility of Gilbert-type effects (Nichol *et al.*, 1964). We shall, therefore, use the relative areas and total protein concentrations to calculate the association constants K_1 and K_2, knowing that at best this can represent values of orders of magnitude and trends. The fact that the distribution of areas under the peaks is apparently unaffected by the total protein concentration would make any K_1 or K_2 calculated a function of total protein concentration. The variation of K_1 and K_2 with total protein concentration would be dependent upon the relative concentrations of the species present. It would be possible, however, to determine the magnitude of the free energy involved in the aggregation reactions since even variation by a factor of 10 in K_1 or K_2 would give a deviation of only 1 kcal in the free energy. Variations of K_1 and K_2 by a factor of 10 would represent the greatest deviation in determination of K_1 and K_2 because of the extremes of the concentration region investigated. The temperature study of sedimentation has been carried out on aliquots of a single sample and, therefore, the concentration of protein at all temperatures investigated is identical except for variations due to changes in the density of water as a function of temperature. This density difference is smaller than the error involved in the determination of the protein concentration. Therefore, at each temperature we have K_1 and K_2 which are intercomparable. The temperature studies of the sedimentation may then be used to calculate the order of magnitude and sign of the ΔH° from $d(\ln K)/dT = \Delta H^\circ/RT^2$, for each reaction, which in turn can be com-

TABLE VII: Thermodynamics of the Association.[a]

Temp (°C)	K_1 (l./mole)	K_2 (l./mole)	ΔF°_1 (kcal/mole)	ΔF°_2 (kcal/mole)	ΔH°_1 (kcal/mole)	ΔH°_2 (kcal/mole)	ΔS°_1 (eu)	ΔS°_2 (eu)
33.8	8×10^4	3×10^2	−7	−3	+24	−11	+100	−26
25	4×10^4	4×10^2	−6	−3.5	+24[b]	−11	+100	−26
9.9	6×10^3	1×10^3	−5	−4	+24	−11	+100	−26
3.1	4×10^3		−4		+24	−11	+100	

[a] Calculated using sedimentation data from temperature study at pH 6.0, $\mu = 0.1$, phosphate. Samples for this study were aliquots from a single large sample of 18.3-mg/ml concentration. [b] Note that ΔH°_1 is the same for temperature study with the protein in cacodylic acid buffer, pH 7.0, $\mu = 0.1$, and ΔS°_1 and ΔF°_1 values would also be similar.

bined with the estimated value for ΔF° obtained from $-RT \ln K$ to give an estimate of the ΔS°. The data for the log K *vs.* $1/T$ plot for the trimer–hexamer interaction at pH 6.0, $\mu = 0.1$, phosphate, and pH 7.0, $\mu = 0.1$, cacodylate, fall on the same line, yielding essentially the same ΔH° and ΔS°. The values for the hexamer–dodecamer interaction are a good deal less certain due to the small areas involved with the 19S species and the relatively small changes in area as a function of temperature.

As the charge on the protein is increased by increasing the pH and deviating from the isoelectric point of this protein (pH 4.7), the sedimentation patterns indicate that the 11S species becomes less and the 7S species more predominant. When the sedimentation patterns are examined at pH 6.0 and above as a function of temperature, it is observed that the 11S or hexameric species is favored at high temperature and the 7S or trimeric species is predominant at low temperature. Specifically, sedimentation at low temperature with the protein near the isoelectric point, pH 5.0, at 10°, demonstrates a predominance of 11S or hexameric species with some 3S or monomer, but no detectable 7S. At pH 9.0, when the protein is highly charged, the sedimentation at 10° indicates the predominance of the trimer or 7S material with some 3S present but no 11S detectable. The sedimentation pattern in the presence of the screening effect of high ionic strength favors the 11S or hexameric material, while decreasing the ionic strength apparently favors the trimer.

Long-range electrostatic or ionic interactions in proteins are generally screened out by higher salt concentrations and are usually increased as we increase the deviation from the isoelectric point. Kauzmann (1959) has discussed the expected pH and ionic strength dependence of protein interactions that are electrostatic and hydrophobic in character. The temperature dependence of electrostatic interactions is generally such that at lower temperatures greater interaction and hence more attraction or repulsion are possible, since there is less competition from the translational motion or kinetic energy to overcome the tendency. One logically could conclude from the pH, ionic strength, and temperature dependence studies that the 7S species is evidently a result of electrostatic interactions. The behavior of the 11S species (its greater prevalence at high ionic strength, high temperature, and when close to the isoelectric point) would seem to favor the proposal that the 11S species or hexamer is formed as a result of screening out of the charge effects and perhaps the subsequent predominance of a specific side-chain interaction between monomers. The trend of the thermodynamics (see Table VII) of the 11S formation would favor the introduction of the hydrophobic interaction mechanism (Scheraga, 1963), although on a strictly thermodynamic basis a charge annihilation mechanism could not be eliminated (Steinberg and Scheraga, 1963). The effect of pH and ionic strength on the 11S species would not appear to favor a charge annihilation mechanism. The mechanism of the interaction in any event would appear to be quite complex with several contributing factors, and with present evidence it appears that the suggestion that hydrophobic force plays the predominant role is acceptable. This certainly does not exclude the appealing possibility of van der Waal's forces playing a role through specific side-chain interactions.

The stability of the 19S species or dodecamer has not been simple to determine. Relative to the 11S species it appears to be favored at higher pH, although on an absolute basis the per cent of 19S decreases with increasing pH (Table I). At higher ionic strength at pH 7.0 the absolute amount of 19S material does not change appreciably; however, since the amount of 11S species increases greatly, the association constant K_2 decreases. At lower ionic strength in phosphate buffer ($\mu = 0.025$) (Table II), the K_2 is also seen to be somewhat smaller than that at pH 7.0, $\mu = 0.1$, phosphate. In pH 6.0, $\mu = 0.1$, phosphate, the K_2 increases with decreasing temperature; however, at pH 7.0, $\mu = 0.1$, cacodylate buffer, the K_2 behavior is not quite as uniform, although the magnitude of the K_2 is quite similar to that at pH 6.0, $\mu = 0.1$, phosphate. These observations lead to the proposal that phosphate buffer ions probably bind to the protein in the various stages of aggregation. The difference between the cacodylate

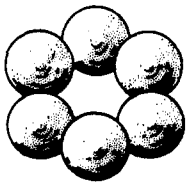

FIGURE 3: Schematic representation of phycocyanin hexamer as concluded from electron micrographs.

buffer, pH 7.0, and the phosphate, pH 7.0, sedimentation behavior also suggests that the trimer binds phosphate ions and this perturbs the trimer–hexamer aggregation.

The reason for the observed maximum in intrinsic viscosity at pH 6.0, $\mu = 0.1$, is at this time not completely understood, although in the pH 5.0–6.0, $\mu = 0.1$, region there is a maximal amount of 11S species. It should be noted that the absorption spectrum, particularly the 622-mμ peak, exhibits a maximum in optical density in the pH 6.0 region at 0.1 μ.[5] The intrinsic viscosity behavior may be interpreted in terms of increased hydration or counterion binding as well as differences in hydrodynamic shape promoted by aggregation. Consequently the intrinsic viscosity effect may not be directly correlated with the spectra or the sedimentation behavior.

Independent electron microscopic studies by Berns and Edwards (1965) are in definite qualitative agreement with the suggested presence of hexameric structure under the conditions essentially identical with those of the present physical studies. The hexamer is demonstrated to be a ring structure as depicted in Figure 3. The monomer and trimer structures are also characterized. The diffusion coefficients and the viscosity data can be analyzed to give an indication of the axial ratios of the hexamer and the other units if the model of a prolate or oblate ellipsoid is used. It is possible to calculate a maximal value of the diffusion coefficient and combine this with the observed diffusion coefficient (Tanford, 1961) to give a ratio of the frictional coefficient, assuming no hydration. This is not a truly desirable procedure since the assumption of no hydration is probably a sizable error. Another procedure is to calculate a β value from the Scheraga–Mandelkern equation (Scheraga and Mandelkern, 1953) by combining a diffusion coefficient with an intrinsic viscosity and an independently determined molecular weight. Alternatively, a sedimentation coefficient and intrinsic viscosity may be used. The intrinsic viscosity from this study cannot in actual fact be simply assigned to any one of the species; however, the intrinsic viscosity at a pH where there is predominance of the hexamer would be most likely characteristic of the hexamer. At pH 5.0, $\mu = 0.1$, where hexamer predominates, $[\eta] = 8.3 \pm 0.3$. Combining this with the diffusion coefficient $D = 4.2 \times 10^{-7}$ and using the molecular weight for the hexamer of approximately 180,000, derived from the minimal molecular weight of 30,000, we arrive at a $\beta = 2.27 \times 10^6$. For a model of a prolate ellipsoid, this would give an axial ratio of approximately 6:1. If the sedimentation coefficient for the hexamer is used, the $\beta = 3.50 \times 10^6$. The high value is no doubt associated with the deviation of the "observed" sedimentation coefficient from the "true" sedimentation coefficient due to the physical interaction discussed earlier. β values for the monomer and trimer species may also be calculated with the proper assumptions; however, these values when interpreted in terms of models are subject to serious deviations or to fortuitous agreement since the $[\eta]$ and S values from this study are not simply interpreted. The β values calculated are all in the 2.0×10^6 to 3.0×10^6 range, which is in general agreement with values observed in other protein systems (Scheraga and Mandelkern, 1953). The ratio of the frictional coefficients calculated from the oversimplified maximal diffusion coefficient method, when used with a prolate or oblate ellipsoid model, gives axial ratios for the hexamer of about 6:1 and 8:1, respectively. The monomer and trimer calculation would be interpreted in the prolate ellipsoid or oblate ellipsoid model with axial ratios very close to one.

The approximate dimensions of the hexamer ring structure from the electron micrographs indicate a ratio of ring diameter/ring thickness of about five to one. This is in substantial agreement with the axial ratios calculated from the diffusion and viscosity data, particularly when one considers the model of the hexagon as derived from the electron micrographs as being annular. The central hole would certainly cause substantial increases in the intrinsic viscosity and a decrease in the translatory diffusion. The diffusion data for the minimal unit can be used to calculate a crude molecular size on the basis of a hard unsolvated sphere model from the use of the Stokes viscosity relationship: $R_E = kT/6\pi\eta D^\circ$. The 3S species gives a molecular size of about 20 A, and the measurements of the electron micrograph would seem to indicate a size of about 30 A for the minimal subunit. The exact dimensions of the monomer are not as yet known. However, the schematic nature of the hexamer is as depicted.

It should be borne in mind that one encounters severe restrictions in attempting to analyze an associating system as complicated as the C-phycocyanin system. The thermodynamic parameters calculated are at best estimates, as are the assigned diffusion coefficients and molecular weights. It is important, however, to indicate also that the proposed equilibria of aggregates appear to be at least qualitatively consistent with the electron microscopic study of C-phycocyanin and other independent investigations of the physical properties of the system (Ó hEocha, 1962; Hattori and Fujita, 1959; Tiselius and Gross, 1934; Goedheer, 1957).

Photosynthetic Aspects. The authors believe that the 11S species is the predominant species *in vivo*. This con-

[5] Dr. J. Bergeron has observed analogous spectral behavior of phycocyanin from *Anacystis nidulans*.

clusion has been reached from the presence of a large amount of this species in crude algal extracts as determined from the ultracentrifuge patterns; from a good deal of what might be at this time termed prejudiced examination of the micromorphology of several blue-green algae (Berns and Edwards, 1965); and, finally, from the compatibility of the proposed structure of the 11S species with the following proposal concerning phycocyanin–chlorophyll interaction and the demonstrated nature of the chlorophyll ordering in the photosynthetic lamella of blue-green algae.

It is generally accepted that phycocyanin plays the role of an additional photoreceptor in photosynthesis and efficiently transfers the absorbed energy to the chlorophyll molecule. The orientation of chlorophyll molecules *in vivo* (Goedheer, 1957) and *in vitro* (Tweet *et al.*, 1964) for maximal energetic efficiency has been described as partly oriented and somewhere near 40 A apart. In blue-green algae it would also necessitate close association with phycocyanin. In the structure elucidated for the 11S species it is proposed that there is a chlorophyll molecule associated with each of the six subunits that make up the hexamer. The porphyrin head sits on the surface of the subunit and the phytol tails could be situated in the hydrophobic center of the hexamer or possibly could be immersed *in vivo* in a lipid fraction of the lamella. This type of association with the phycocyanin would permit a semiordered array as proposed by Goedheer (1957) and an optimal separation of chlorophyll molecules. The proposal of the chlorophyll–phycocyanin complex is not intended to suggest that this is the only situation in which one finds chlorophyll in the blue-green algae. It is merely a proposed mechanism for optimal energy transfer which is consistent with the several observed properties of the chlorophyll and phycocyanin.

It is interesting to note that the phycocyanin fluorescence depolarization measurement of Goedheer (1957) indicates internal inductive energy transfer for phycocyanin in the pH 4.0–6.0 region. This is the region of greatest stability of the hexamer structure, and the hexamer structure certainly would favor internal inductive energy transfer between subunits and subsequent fluorescence from the differently oriented chromophores. At pH 7.0 and above, the depolarization decreases, which is consistent with our view that the monomer and trimer units are favored at the higher pH. Probably one or possibly two chromophoric tetrapyrroles are associated with each minimal unit. Therefore, the trimer and monomer would not be as effective as the hexamer in depolarization because of inductive energy transfer to randomly oriented chromophores. The fluorescence depolarization measurements, therefore, suggest that the pH 4.0–6.0 stable structure, which we propose as the hexamer, is the most efficient structure for energy transfer to the chlorophyll molecules. It seems unlikely that superposition of the chlorophyll porphyrin head and the phycocyanin chromophore would be necessary for the proposed model to be efficient.

It would thus appear that a phycocyanin–chlorophyll aggregate is feasible, consistent with evidence contributed concerning chlorophyll orientation and with the structure of what the present authors suggest to be the predominant phycocyanin structure *in vivo*. It can also be seen that this type of chlorophyll phycocyanin aggregate would be particularly labile to normal extraction for at least two reasons: first, since the phycocyanin equilibria in aqueous solution would permit the chlorophyll molecules to split off the phycocyanin and deposit as insoluble chlorophyll; and, second, if the phytol tail should be imbedded in lipid instead of in the center of the hexamer, the phycocyanin would be expected to be present in the crude extracts in solution, while the chlorophyll might associate with the hydrophobic lipid fraction. Experiments designed to isolate or synthesize this proposed aggregate are needed.

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Kinetics of Iodination. IV. A Comparison of the Kinetics of Iodination of L-Tyrosine and Some Derivatives*

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ABSTRACT: The kinetics of iodination of L-tyrosine and some derivatives has been studied spectrophotometrically in an aqueous buffered system. The second-order rate constant k for the iodination of L-tyrosine was 5.73×10^3 l. mole^{-1} sec^{-1}. On the basis of the rate constant for tyrosine being 100, the relative rates of iodination for the derivatives were glycyl-L-tyrosine, 162; glycyltyrosylglycine, 134; L-tyrosyl-L-tyrosine, 110; L-tyrosine methyl ester, 77; *N*-acetyl-L-tyrosine, 74; *N*-acetyl-L-tyrosine methyl ester, 61; *N*-acetyl-L-tyrosine ethyl ester, 55; 3-iodo-L-tyrosine, 6; and *N*-acetyl-3-iodo-L-tyrosine, 3. *O*-Methyl-L-tyrosine did not iodinate under the conditions studied. The Arrhenius activation energy E_a in the iodination of the iodinated derivatives was 21 kcal mole^{-1}, while all the other compounds had values of 16–17 kcal mole^{-1}. The kinetics of iodination of all the compounds was compatible with the concept of iodination occurring by way of phenolate anion and molecular iodine through a quinoid intermediate.

The kinetics of iodination of *N*-acetyl-L-tyrosine and *N*-acetyl-3-iodo-L-tyrosine has been studied previously. The rate for iodination of *N*-acetyl-L-tyrosine exceeded that of the iodinated derivative by a factor of 20–30 over the pH range 5.40–9.80 (Mayberry *et al.*, 1964). The kinetic data were interpreted to indicate that the reactive species in phenolic iodination were molecular iodine and phenolate anion and, further, that iodination proceeded *via* a quinoid intermediate. The reactions are subject to general base catalysis, and the function of the base is viewed as proton removal in the "rate-limiting" step from the quinoid intermediate (Mayberry and Bertoli, 1965).

The present study was initiated to determine the effect of the peptide linkage and of the ionizable groups of L-tyrosine upon the rates and activation energies of iodination. Such comparisons may have relevance to *in vivo* iodination of tyrosine within the thyroglobulin molecule.

Experimental

Materials. Recrystallized *N*-acetyl-L-tyrosine, *N*-acetyl-3-iodo-L-tyrosine, and *O*-methyl-L-tyrosine were gifts of Dr. R. Pitt-Rivers. Recrystallized glycyl-L-tyrosine, glycyl-L-tyrosylglycine, L-tyrosine methyl ester, *N*-acetyl-L-tyrosine methyl ester, *N*-acetyl-L-tyrosine ethyl ester, 3-iodo-L-tyrosine, and L-tyrosine were given by Dr. H. Cahnmann. L-Tyrosyl-L-tyrosine was purchased from Cyclo Chemical Corp., Los Angeles, Calif. High voltage electrophoresis in Barbital buffer at pH 8.68 revealed each compound to run as one spot as detected by the ferric chloride–potassium ferricyanide reagent for phenols (Barton *et al.*, 1952). The *O*-methyltyrosine does not stain with the latter, but this serves as a means for detecting trace impurities of phenolic substances in the compound. In addition, the purity of the *O*-methyltyrosine was further determined by ultraviolet spectra in acid and base, a method particularly advantageous for determining impurity in *O*-methyltyrosine (Wetlaufer *et al.*, 1958).

The iodine, potassium iodide, sodium chloride, sodium carbonate, and sodium bicarbonate were the best commercially available reagent grade chemicals. Water redistilled in glass was used in all experiments.

Kinetic Runs. All reactions were performed in a carbonate buffer system containing 0.120 M sodium bicarbonate and 0.075 M sodium carbonate at pH 9.60. Ionic strength was maintained at 0.64 by the addition of sodium chloride which was 0.097 M in the system. The stoichiometric concentration of tyrosine or derivative, iodine, and iodide was constant in each run at 2×10^{-4} M, 5×10^{-5} M, and 0.193 M, respectively. Each run was

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