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Ultracentrifuge Investigation of Protein Aggregation in Dilute Solutions of C-Phycocyanin*

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ABSTRACT: Sedimentation analysis of dilute solutions of phycocyanin with absorption optics demonstrated that aggregates previously detected at concentrations up to 40 mg/ml are present at concentrations as low as 0.2 mg/ml at pH 5–9. The S values of the various aggregates also agree with results in previously reported work with concentrated solutions and therefore permit the interpretation of absorption measurements and electron microscopy in terms of these aggregates. Underexposure of photographic plates with the absorption optics on the ultracentrifuge is characterized as being responsible for the probable inability of Hattori *et al.* [Hattori, A., Crespi, H. L., and Katz, J. J. (1965), *Biochemistry 4*, 1225] to detect higher aggregates. The photographic absorption technic, therefore, requires close scrutiny of exposures for proper application.

In previous reports, Scott and Berns (1965; Berns and Scott, 1966) demonstrated that C-phycocyanin from several *Cyanophyta* aggregates reversibly. The aggregation was studied in the concentration range of 4–50 mg/ml as a function of temperature, pH, and ionic strength. Reversible aggregation of this protein was characterized as being sensitive to the use of cellulose ion exchangers and certain types of calcium phosphate preparations (Scott and Berns, 1965). Aggregates sedimenting at 11 S and higher were described as being important *in vivo*. The 11S material was suggested to be a hexamer and hydrodynamic measurements and electron microscopy (Berns and Edwards, 1965) agreed quite well in delineating the probable size and shape of this aggregate. Most of the physical measurements in these studies have been at concentrations of 5 mg/ml and greater; however, spectrophotometric and electron microscopy studies were generally confined to concentrations in the 0.5-mg/ml region and lower. The question arises, therefore, as to whether absorption spectra can be interpreted in terms of the aggregates proposed from work with higher concentrations and are the electron microscopy results relevant to the species observed at much higher concentration. If simple equilibria exist, then a natural consequence, judging from the published data for this system, should be an increase in concentration of the slower sedimenting species as the total protein concentration is decreased. Hattori *et al.* (1965) have reported that in ultracentrifugation of dilute solutions the slower sedimenting 3S material is favored and in most cases the 7S or 11S species is totally absent. These studies were performed with phycocyanin purified by ion-exchange chromatography and, therefore, the aggregation phenomenon as investigated is an artifact (Scott and Berns, 1965). Even in this type of system, however, immunochemical techniques (Scott and Berns, 1965) detect the presence of higher aggregates. Ouchterlony double diffusion utilizes antigen concentrations of 0.5 mg/ml and lower. A serious inconsistency that bears quite strongly on studies of

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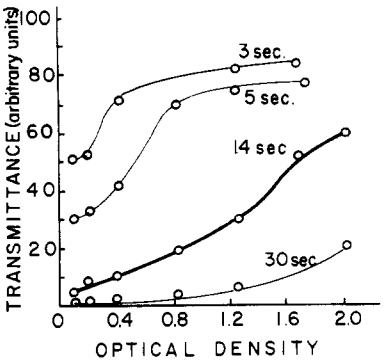


FIGURE 1: Tyrosine calibration of absorption optics as a function of exposure time. Tyrosine solutions obey Beer's law at 278 mμ when examined in a Beckman DB spectrophotometer. The optical densities refer to a 1-cm light path. The optical density, therefore, also represents concentration.

biliproteins *in vivo* and *in vitro* thus exists. A systematic investigation of sedimentation behavior in dilute solutions as a function of pH and method of purification was, therefore, undertaken to characterize the species present in concentrations of less than 1 mg/ml.

Experimental Section

Phycocyanin from the *Cyanophyta Plectonema calothricoides* was used in all experiments. The cell lysis and purification of the phycocyanin were performed as previously reported (Scott and Berns, 1965). A parallel purification of phycocyanin was carried out with pH 6.0, $\mu = 0.1$, phosphate buffer in all steps including cell lysis. Purity of the phycocyanin was checked by Sephadex (G-100) gel filtration. An aliquot of purified phycocyanin was examined under conditions that would separate allophycocyanin from C-phycocyanin (pH 7.15, $\mu = 0.01$, phosphate buffer at 4°) and only small amounts of allophycocyanin were detected. The purified protein was stored under 50% saturated ammonium sulfate at 4° prior to use. Aliquots were spun down and decanted, appropriate buffer was added to the precipitate, and the sample was dialyzed overnight at 4° with two buffer changes. The initial concentration was estimated by 278-mμ OD and was then adjusted if necessary by buffer dilution. The protein concentration was determined by a semimicro-Kjeldahl procedure on duplicate aliquots (Kabat and Mayer, 1961). A Spinco Model E ultracentrifuge, equipped with an ultraviolet absorption optical system and RTIC, was used for the sedimentation analysis. Sedimentation was always at 59,780 rpm and the temperature was maintained close to 25°. The collimating and condensing lenses of the ultraviolet system were cleaned before each day's experiments. Kodak contrast process Ortho sheet film was used and a very detailed study of exposure times was undertaken. Exposure times of 3–40 sec were taken on a single sedimentation experiment. Great care in developing and handling procedures was also observed. The exposed film was developed by the professional photographic staff of this laboratory, using solutions carefully controlled at 20°. The film was placed in fresh Kodak D-11 developer for 5 min, followed by a 0.5-min wash with agitation, then fixed for 5 min, with continual agitation during the first minute. It was then washed with agitation for 0.5 min and a hypoclearing agent was used for 1–2 min, followed by a 5-min wash and use of "photoflow" according to the manufacturer's directions. The film was then dried.

The optical densities of the protein samples examined in the ultracentrifuge were all scanned at 278 mμ in a Beckman DB spectrophotometer usually in a 1-cm cell prior to use. These optical densities varied from 0.61 to 1.70. Calibration of the response of the entire absorption optical system was undertaken by the examination of solutions of tyrosine in distilled water. The tyrosine solutions were examined in the DB spectrophotometer; they had optical densities varying from 0.1 to 2.0 and were found to obey Beer's law. The tyrosine samples were then examined on the ultracentrifuge at 59,780 rpm with varying exposure times (3, 5, 14, and 30 sec). The developed negatives for the protein experiments and the tyrosine calibration experiments were examined with a Hilger–Watts microdensitometer kindly made available to us by Dr. G. Janz, Chemistry Department, Rensselaer Polytechnic Institute.

The evaluation of microdensitometer tracings is complicated by several factors. In general, one cannot assume that the densitometer readings are proportional to the solution optical density or concentration. The response will be a curve and relative concentrations and even the calculation of inflection points may be in error (Strong, 1938). A plot of tyrosine optical density (or concentration) *vs.* microdensitometer transmittance readings as a function of film exposure time in Figure 1 demonstrates that at 3 and 5 sec there is a somewhat linear response, with fair sensitivity at only low optical densities. The 30-sec exposure time is not sensitive except at very high optical densities. The 14-sec exposure time does deviate from perfectly linear optical density *vs.* transmittance behavior; however, it shows close to linear behavior with good sensitivity over the entire optical density region investigated. This would appear to demonstrate that a study of exposure time is of critical importance in the evaluation of the presence or absence of sedimenting species by dilute solution absorption measurements. It would also appear to indicate that for the 0.61–1.70 optical density range investigated in these protein solutions 14-sec exposures are near the optimal conditions for such evaluation.

Several phycocyanin samples were also examined during this investigation at higher concentrations with the schlieren system, to determine that they were reproducible with the data of Scott and Berns (1965). Schlieren patterns were analyzed with the aid of a Nikon microcomparator to measure distances traversed as a function of time. The absorption pictures were analyzed by measurements from the tracing obtained

with the Hilger–Watts microdensitometer. Sedimentation coefficients evaluated from the density scans were somewhat less precise than those calculated from schlieren photographs. This was due to difficulties in reproducing the location of the midpoint of the front of each sedimenting species from exposure to exposure. Although the presence of the several species was easily ascertained, the precise values of the sedimentation coefficients, particularly for minor components, should be considered as no better than $\pm 10\%$. Greater precision may be obtained with considerable effort and the analysis of a very large number of densitometer scans. For the purposes of this investigation, it was deemed unnecessary to attempt a more precise determination of sedimentation coefficients. Sedimentation coefficients were corrected to S_{25} values but no effort was made to extrapolate values to infinite dilution since a great deal more data at each pH would be needed in the dilute region to justify an extrapolation. Attempts to calculate the relative concentration of each species from the density scans were deemed too speculative to be of value. Instead the evaluation of components as major or minor was used.

Criteria for the establishment of equilibrium in the phycocyanin system were considered in great detail in all our studies. Samples of phycocyanin were dialyzed for up to 4 days at 4° with daily buffer changes; the schlieren ultracentrifuge sedimentation patterns were identical with those for a sample dialyzed overnight with several changes of dialysate. Phycocyanin at pH 7.0, $\mu = 0.1$, was dialyzed into pH 7.0, $\mu = 0.01$ buffer, examined after 1 day, and allowed to sit in the cold room for an additional 3 days No changes in ultracentrifuge behavior were noted. A sample of fully deuterated phycocyanin was lyophilized from pH 6.0, $\mu = 0.1$, buffer twice and reconstituted. The distribution of 19S, 11S, and 7S material from a schlieren pattern was essentially identical with that of the original material with the exception that the 19 S appeared to have a small increase in area and asymmetry. Shifts in the sedimentation pattern of the protein upon a change of 10° in the temperature at which sedimentation was carried out, indicated a fast rate of equilibration (Scott and Berns, 1965). At the particular temperature, equilibrium was apparently established within 1 hr. Changes in ionic strength and pH also resulted in rapid equilibration. Close examination of sedimentation patterns appeared to indicate that the schlieren patterns between peaks return to the base line and changes in angular velocity apparently do not affect the relative areas of the several peaks (Scott and Berns, 1965). These results apparently preclude rate constants for the system which are in competition with the rate of sedimentation (Nichol *et al.*, 1964). All these factors seem to indicate that there is not a very slow rate of attainment of equilibrium. Ouchterlony immunodiffusion studies made over a period of several days resulted in sharp lines difficult to explain if this system were equilibrating over this same period of time. Sephadex gel filtration on G-100 and G-200 columns resulted in incipient resolution of the several species involved but complete resolution is not possible at present, apparently because of reequilibration during the gel filtration. This result is also indicative of a relatively fast attainment of equilibrium.

Consideration of all these facts led us to interpret the results in terms of a system that is at equilibrium and is the simplest type of interacting system as classified by Nichol *et al.* (1964). Experiments in progress with the separation type of analytical ultracentrifuge cell yielded results consistent with the proposal that equilibration is fairly rapid; however, the experiments also indicate the possible existence of complex Gilbert type effects. The interpretation must await further results. This does not, however, affect the interpretation of the results for the present study since we are concerned only with the proposal of the presence of higher aggregates in very dilute solutions. In this investigation we have observed precautions consistent with those of other workers (*i.e.*, Hattori *et al.*, 1965) for attainment of equilibrium. The results of this study, therefore, should be comparable to those in other published reports whatever the nature of the complex reaction process when it is finally resolved.

Results

In Tables I and II typical examples of data for each pH are presented. Several experiments for each pH gave similar results. The precision of the S values was no better than approximately 10%; however, this was in general due to the procedure that must be followed in analyzing the absorption plates. The component present in the greatest abundance was usually characterized with better precision with the absorption procedure. The same samples of phycocyanin examined under pH and concentration conditions similar to those described by Scott and Berns (1965), but with schlieren optics, gave results similar to theirs. Phycocyanin samples examined at an ionic strength of $\mu = 0.01$ and 0.02 and pH 6.0 or 7.0, had an increased amount of 7S material with 11S and 19S species still present. These studies were performed at higher protein concentrations and the results were in agreement with those reported by Scott and Berns (1965).

Exposure times for the absorption studies were very critical. In a given sedimentation experiment, when exposure times from 3 to 40 sec were used, it was possible to analyze the resulting photograph and find apparently widely differing results. Scans of exposures in the 5-sec range exhibited only one sedimenting species, a very slow one, usually 3 or 7 S. A 15- or 20-sec exposure taken within a few minutes of the 5-sec exposure in the same experiment demonstrated the existence of two or more species depending on the pH (see Figure 2 for example of this procedure). Furthermore, the data from the 5-sec exposure in a particular experiment coincided exactly with the distance traversed as a function of time for the slowest sedimenting species from the 15- or 20-sec exposure. This demonstrates that underexposures in a sedimenting system with multiple components result in the apparent presence of a single

TABLE I: Typical Sedimentation Experiments.

pH[a]	Concn (mg/ml)	S_{25}	Rel % of Component[b]	Extrapolated $S_{0,25}$[c]	Rel % of Component[d]
				3.7	6
5.0	0.22[e]	5.7	Minor	6.3	10
		10.3	Major	12.1	75
				18.9	9
6.0	0.26	8.8	Minor	6.2	25
		12.3	Major	12.6	68
		21.9	Possibly minor?	19.2	8
7.0	0.47	7.0	Major	7.0	62
		15.6	Minor	12.9	32
		25.8	Possibly minor?	20.8	7
8.0	0.53	5.8	Major	6.5	65
		9.4	Minor	12.5	31
		26.4	Possibly minor?	20.1	5
9.0	0.53	2.6	Major	5.2[f]	93
		14.9	Minor	18.9	7
7.0[g]	0.55	7.1	Major		
		13.4	Minor		
6.0[g]	0.32	11.2			

[a] All ionic strength = 0.1, and all were purified at pH 7.0. [b] Evaluated from relative density on microdensitometer tracing. [c] Data from Scott and Berns (1965). [d] Data from Scott and Berns (1965) by integration of area under schlieren peaks. [e] Material (19 S) detected in samples of slightly higher concentration. Species (3 S) is detected at this and all other pH value when very low photographic exposure times are used. [f] Trailing edge noted. [g] Hydroxylapatite-treated preparations.

TABLE II: Sedimentation Coefficients from pH 6.0 Purified Phycocyanin.

pH[a]	Concn (mg/ml)	S_{25}
5	0.578	7.9
		12.0
		18.5
5	~20.0	4.1
		7.4
		12.2
		19.4
		27.2
6	0.55	6.04
		12.2
		16.9
6	~20.0	6.5
		12.3
		17.6
8	~20.0	5.9
		11.5

[a] All ionic strength = 0.1.

sedimenting species, usually the slowest. This result was found at all pH values and also with hydroxylapatite-treated pH 7.0 samples. The S_{25} values from the absorption experiments were in general agreement with the extrapolated values from Scott and Berns (1965). Certainly substantial deviations are evident in values cited in Table I; however, these are apparently due to random error in the calculation process. There is no trend of decreasing S_{25} value with decreasing concentration.

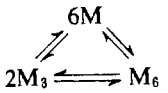
Sedimentation analysis at pH 5.0, $\mu = 0.1$, of samples of high concentration purified at pH 6.0, $\mu = 0.1$, showed the presence of five species (see Table II). A much larger amount of 19S material and the presence of a 27S species were apparently a result of the lower pH purification. The 27S and 3S species were by far the smallest in concentration. The same sample analyzed at high dilution by absorption optics demonstrated only the apparent presence of three species, 7, 11, and 19 S. The two smallest components, 3 and 27 S, were possibly present in too great a dilution to be characterized under these conditions or it is possible that the effect of dilution on the equilibria was responsible.

The pH 6.0 purified material when exposed to pH

8.0 did not exhibit the presence of 19 S or higher sedimenting material. When a sample exposed to pH 7.0 or higher was reequilibrated at pH 5.0, no 27S material was detected and very little 19S material. This finding is consistent with previous suggestions (Scott and Berns, 1965; Berns and Scott, 1966) about the possibility of electrostatic repulsion being responsible for degradation of aggregates important *in vivo*.

Discussion

The results demonstrate that the several sedimenting species present at high concentrations in the phycocyanin system at pH 5.0–9.0 were definitely present at concentrations as low as 0.2 mg/ml and possibly lower.[1] Hydroxylapatite-treated samples also contained the same species at high and very low concentrations. The relative concentration of the several species was more difficult to ascertain. Some increase in the relative concentration of the smaller species was detected at lower concentrations. The displacement was not nearly what would be expected if the simple equilibria $6M \rightleftharpoons 2M_3 \rightleftharpoons M_6$ as earlier proposed and used in interpretation of this system were correct (Hattori *et al.*, 1965; Scott and Berns, 1965). Careful examination of the phycocyanin system from normal *Cyanophyta* and from the thermophilic *Synechococcus lividus* phycocyanin (Berns and Scott, 1966) presented data that argue against the simple equilibria as presented. It was found that near the isoelectric point at pH 5.0 at 8° only 3S and 11S material were present; at pH 9.0 at 8° only 3S and 7S were present. This led to the proposal (Berns and Scott, 1966) that monomer polymerizes to form hexamer and the electrostatically favored trimer forms only from hexamer as a result of repulsive forces. The aggregating system, therefore, might be better represented by



At pH values deviating from the isoelectric point the role of the trimer in this equilibrium becomes nebulous since its ability to form hexamer or monomer must necessarily be affected by the charging effect of higher pH.

The existence of higher aggregates in dilute solutions and the lack of appearance of large concentrations of smaller species are not observations that would be predicted from a simple interpretation of an aggregat-

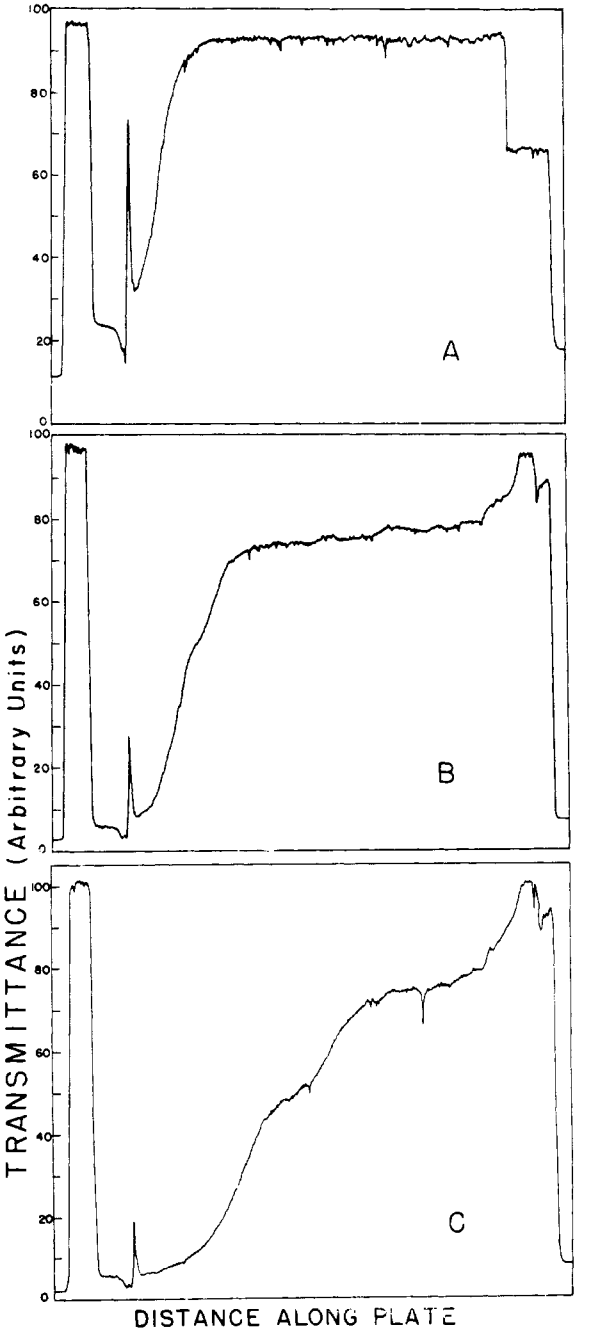


FIGURE 2: Typical microdensitometer tracing of absorption photographs. It demonstrates effect of underexposure of photographic plates on resolution of sedimenting species present. Sedimentation is from left to right at 59,780 rpm; sharp peak at left is solution meniscus. For details of interpretation of such a tracing, see Schachman (1959). Sample is at pH 7.0, μ = 0.1, with a protein concentration of 0.47 mg/ml, optical density at 278, mμ = 0.95. (A) Time of sedimentation, 21 min; 5-sec exposure. Note only one inflection detectable, decrease at right end is due to sensitivity adjustment. (B) Time of sedimentation, 23 min; 14-sec exposure. Note two components are already evident. (C) Time of sedimentation, 48 min; 14-sec exposure. Resolution of two components clearly evident.

[1] After completion of this work and submittance of this manuscript several samples of phycocyanin were examined by Dr. D. Yphantis using a Beckman-Spinco Model E ultracentrifuge equipped with a photoelectric-scanning system and monochromator. Using conditions outlined in this paper, Doctor Yphantis confirmed the presence of 7S, 11S, and 19S species in dilute solutions. The samples examined had an optical density in the 278-mμ region of about 0.60. A sample examined at pH 6.0 contained as much as 20% 19S material. We are grateful for Doctor Yphantis' kindness in examining the phycocyanin samples.

ing system. They have led us to consider the possible lack of attainment of equilibrium in this system. For the present, this possibility is not supported by the experimental detail cited earlier in this study. The complex nature of this system, which permits higher aggregates at very low concentrations, is still to be elucidated.

The presence of all the previously reported aggregates in the low concentration region as in the more concentrated solutions, permits the interpretation of spectroscopic measurements and electron microscopy and other work with dilute solutions in terms of these aggregates. The results of this study would also appear to be in direct conflict with those of Hattori *et al.* (1965), in which they report the essential disappearance of higher aggregates at low concentrations. A great deal of interpretation of difference spectra is based upon the existence of preponderantly monomer at concentrations of the order of 0.2 mg/ml and lower. Although our experimental conditions were not all identical with those of Hattori *et al.*, in that we usually chose to work at higher ionic strengths, we found that even at 0.01 ionic strength the equilibria were never sufficiently different at pH 7.0 to account for more than a 15% increase at most in 7S species because of less effective screening of the electrostatic repulsion. At pH 5.0, which is close to the isoelectric pH where the charging is quite a bit less, the difference between 0.1 and 0.01 ionic strength is barely significant as would be predicted. It is highly probable, therefore, that the absorption ultracentrifuge work of Hattori *et al.* with dilute solutions is erroneous simply because of underexposure of photographs. Consequently, the interpretation of their difference spectra is quite suspect.

It is apparent that the use of the photographic ultraviolet absorption system of the Spinco Model E ultracentrifuge for work with dilute solutions of macromolecules is quite valuable for establishing the presence or absence of species in aggregating system. However, it is important to scrutinize carefully the exposures used. Furthermore, minor components may or may not be characterized from the absorption photographs since the characterization depends on relative changes in the optical density that may be difficult to detect because of limitations in the contrast available in the film used.

Acknowledgment

We wish to thank Dr. G. Kegeles for a stimulating discussion of some of the problems involved in complex equilibria in aggregating systems.

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