hydrogen bonds are formed and hence *inter*molecular hydrogen bonds, leading to gelation, can form. The time dependence of the gelation process and the effect of various solution variables on it is under further study, and we hope to be able to report further results in the near future.

References

Aschaffenburg, R., and Drewry, J. (1957), *J. Dairy Sci. 180*, 376.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Christensen, L. K. (1952), *Compt. Rend. Trav. Lab. Carlsberg 28*, 37.

Herskovits, T. T., Townend, R., and Timasheff, S. N. (1964), *J. Am. Chem. Soc. 86*, 4445.

Moffitt, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Nakanishi, K. (1960), *Bull. Chem. Soc. Japan 33*, 793.

Nozaki, Y., and Tanford, C. (1965), *J. Biol. Chem. 240*, 3568.

Schachman, H. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.

Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G. Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Tanford, C., Buckley, C. E., De, P. K., and Lively, E. P. (1962), *J. Biol. Chem. 237*, 1168.

Tanford, C., De, P. K., and Taggart, V. G. (1960), *J. Am. Chem. Soc. 82*, 6028.

Tanford, C., and De, P. K. (1961), *J. Biol. Chem. 236*, 1711.

Timasheff, S. N., and Susi, H. (1966), *J. Biol. Chem. 241*, 249.

Timasheff, S. N., and Townend, R. (1961), *J. Am. Chem. Soc. 83*, 470.

Timasheff, S. N., and Townend, R. (1962), *J. Dairy Sci. 55*, 259.

Timasheff, S. N., and Townend, R. (1965), *Biochem. Biophys. Res. Commun. 20*, 360.

Timasheff, S. N., Townend, R., and Mescanti, L. (1966), *J. Biol. Chem. 241*, 1863.

Townend, R., Weinberger, L., and Timasheff, S. N. (1960a), *J. Am. Chem. Soc. 82*, 3175.

Townend, R., Winterbottom, R. J., and Timasheff, S. N. (1960b), *J. Am. Chem. Soc. 82*, 3161.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Dissociation and Association of Phycocyanin*

Eiji Fujimori and Joseph Pecci

ABSTRACT: Phycocyanin from *Anacystis nidulans* dissociates when treated with *p*-mercuriphenylsulfonic acid. Upon removal of the mercurial with glutathione, reassociation occurs. The dissociation is incomplete at pH 5.0 giving rise to various dissociated forms. The dissociation at pH 7.0 is almost complete, yielding two types of subunit which can be separated by gel filtration. The major subunit is characterized by its absorption maximum at 605 mμ and the minor subunit by two maxima at 615 and 652 mμ. The dissociation of phycocyanin results in a decrease of its 620-mμ absorption maximum as well as a shift of it to shorter wavelengths.

Its fluorescence is also decreased. Reassociation of the dissociated subunits brings about a partial regeneration of this decrease in absorption and fluorescence. These reversible spectral changes occurring during dissociation–association are attributed particularly to changes taking place in a highly fluorescent pigment present in the major subunit. Another pigment in the major subunit appears to be less sensitive to conformational changes of the protein.

It has been known ever since the investigations of Svedberg *et al.* (1928, 1929, 1932) and Eriksson-Quensel (1938) that the association–dissociation of phycocyanin, one of the accessory photosynthetic chromoproteins of the red and blue-green algae, is pH dependent. Recent investigations have not only confirmed this (Hattori and Fujita, 1959; Berns *et al.*, 1963), but have also shown a dependence of the reversible aggregation on ionic strength and temperature (Berns and Edwards, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965; Berns and Scott, 1966). Under conditions of varying pH and ionic strength, the association–dissociation of phycocyanin is accompanied by characteristic changes in the absorption and fluorescence spectra (Bergeron, 1963; Goedheer and Birnie, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965). Urea and sodium dodecyl sulfate are capable of causing phycocyanin to dissociate into smaller subunits (Berns *et al.*,

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TABLE I: Sedimentation Coefficients, $s_{20,w}$ of Phycocyanin.[a]

	Monomer	Trimer	Hexamer		Dodeca-mer	Higher Aggregates
pH 5.0						
Untreated			9.2	—	17.1	—
Treated (PMPSA)	3.5		10.1	(11.7)[b]		
Treated (PMPSA + glutathione)			9.3	13.3	16.5	(~20)[b]
pH 7.0						
Untreated		5.5	(10.2)[b]		16.4	
Treated (PMPSA)	5.0					
Treated (PMPSA + glutathione)		5.5	(9.3)[b]		16.8	

[a] $s_{20,w}$ values were calculated from a single determination (see Figures 1–4) not from extrapolated values. [b] Values in parentheses denote that some difficulty was experienced in accurately locating the apex of the schlieren peak due to its broadness.

1964). As a result of these recent investigations, it has been proposed that the association–dissociation equilibria of phycocyanin involves the monomer, trimer, and hexamer as well as other higher aggregates.

Previous studies in this laboratory (Fujimori and Quinlan, 1963) have shown that *p*-mercuribenzoate produces a decrease in the absorption and fluorescence of phycocyanin at pH 7.0. This decrease is partially recovered upon the addition of glutathione. Recent investigations (Fujimori, 1964; E. Fujimori and J. Pecci, 1966, unpublished data) on phycoerythrin, another chromoprotein of the red algae, have demonstrated that *p*-mercuribenzoate can cause phycoerythrin to dissociate into smaller subunits. In this paper, phycocyanin from the blue-green alga *Anacystis nidulans* is shown to undergo reversible dissociation when treated with *p*-mercuriphenylsulfonic acid (PMPSA).[1] The reversibility is demonstrated by subsequent treatment with glutathione. Sedimentation analyses were carried out on the phycocyanin system both at pH 7.0 and 5.0. Changes in absorption and fluorescence of the system were also investigated. It was possible, at pH 7.0, to effect a separation of the dissociated subunits by using a gel filtration technique. The spectral characteristics of these isolated subunits were investigated.

Materials and Methods

Chemicals. *p*-Mercuriphenylsulfonic acid (sodium salt) was obtained from the Sigma Chemical Co. The glutathione (reduced) was obtained from Calbiochem. The Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Inc.

Algal Cultures. Cultures of *A. nidulans* Strain No. M-2.1.1 were obtained from the Kaiser Foundation Research Institute. *A. nidulans* was grown in BGM[2] medium[2] at 37° while 1% carbon dioxide in air was continuously bubbled through the culture. Constant illumination was provided by a 400-w, cool white fluorescent lamp.

Preparation of Phycocyanin. The algal cells were harvested by centrifuging at 5000*g* for 10 min. The residue was washed twice with 0.1 M acetate buffer, pH 5.0. After the final washing, the cells were resuspended in the same buffer. Portions (50 ml) of the suspension were insonated for 20 min each using a Raytheon sonic oscillator, Model DF-101 (250 w, 10 kcycles). The insonated suspension was centrifuged at 30,000*g* for 60 min at about 5° to remove the bulk of insoluble material. The supernatant liquid was recentrifuged at 100,000*g* for 60 min. The final supernatant liquid was made 50% saturated with ammonium sulfate and the resulting precipitate of crude phycocyanin was collected by centrifuging at 50,000*g* for 30 min. The precipitate was dissolved in a minimal amount of 0.1 M acetate buffer, pH 5.0, and centrifuged at 100,000*g* for 15 min. The supernatant liquid was made 25% saturated with ammonium sulfate, stored overnight at 4°, and finally centrifuged at 50,000*g* for 30 min. The precipitate was dissolved in a minimal amount of 0.1 M acetate buffer, pH 5.0, and centrifuged at 100,000*g* for 15 min. The supernatant liquid was dialyzed against two 5-l. changes of the same buffer. Phycocyanin prepared in this manner showed a ratio of optical density at 620:280 mμ of 4.4. To obtain phycocyanin at pH 7.0, phycocyanin in 0.1 M acetate buffer, pH 5.0, was dialyzed overnight against 0.1 M phosphate buffer, pH 7.0.

Determination of Phycocyanin Concentration. Aliquots of the phycocyanin solution were dialyzed overnight against distilled water to remove buffer salts. The dialysates were quantitatively transferred to tared, pretreated flasks, and lyophilized. Residues were further dried in a vacuum desiccator over Drierite to constant weight.

Gel Filtration. A column (1.5-cm diameter, 25-cm length) of Sephadex G-100 was used. Eluted fractions

[1] Abbreviations used: PMPSA, *p*-mercuriphenylsulfonic acid.

[2] Allen, M. B. (1963), list of cultures maintained by the Laboratory of Comparative Biology, Kaiser Foundation Research Institute, Richmond, Calif.

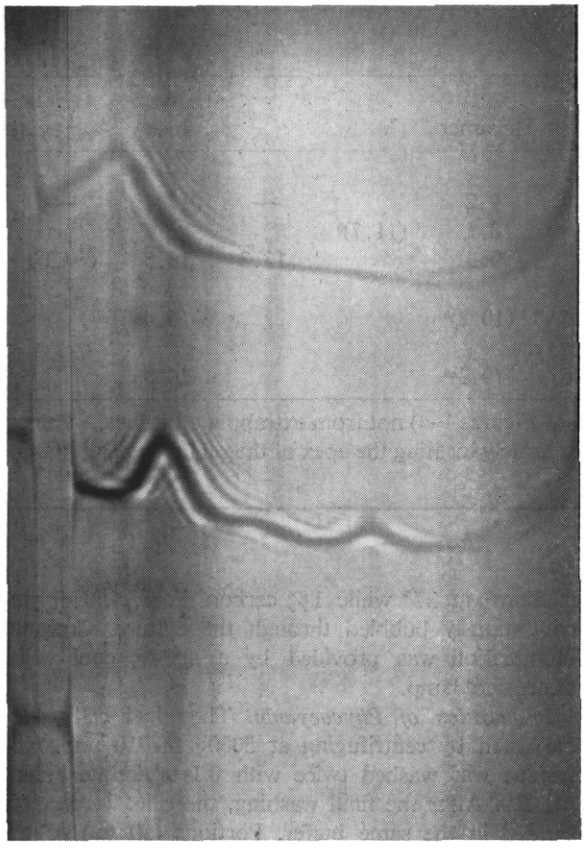

FIGURE 1: Effect of PMPSA (10^{-3} M) on the sedimentation pattern of phycocyanin (0.36%) at pH 7.0 (0.1 M phosphate buffer, 1% NaCl). Lower sedimentation pattern: untreated phycocyanin; upper pattern: phycocyanin treated with PMPSA. Photographs were taken 28 min after the centrifuge reached 59,780 rpm. Menisci are on the left; temperature, 20°; phase-plate angle, 60°.

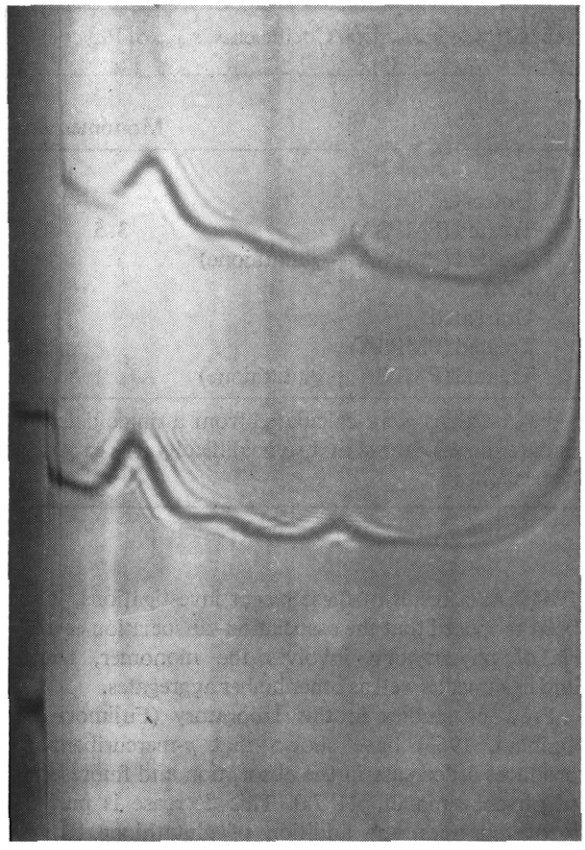

FIGURE 2: Effect of glutathione (5×10^{-3} M) on the sedimentation pattern of phycocyanin (0.36%) untreated and treated with PMPSA (10^{-3} M) at pH 7.0 (0.1 M phosphate buffer, 1% NaCl). Lower sedimentation pattern: untreated phycocyanin plus glutathione; upper pattern: PMPSA-treated phycocyanin plus glutathione. Photographs were taken 28 min after the centrifuge reached 59,780 rpm. Menisci are on the left; temperature, 20°; phase-plate angle, 60°.

of 1 ml each were collected using a Research Specialties Co., Model 1205 automatic fraction collector. Each fraction was diluted with the appropriate buffer solution to 3.5 ml for the measurement of absorption spectra.

Sedimentation Analysis. Sedimentation studies were made at 20° and 59,780 rpm using a Spinco Model E analytical ultracentrifuge. Schlieren optics and 12-mm cells were employed. Standard procedures were used for the determination of sedimentation coefficients. No corrections were made for the presence of 10^{-3} M PMPSA and/or 5×10^{-3} M glutathione. A partial specific volume of 0.75 as reported for phycocyanin from *Ceramium rubrum* (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929) and *Plectonema calothricoides* (Scott and Berns, 1965) was used in all calculations.

Optical Measurements. Absorption spectra were measured with a Cary Model 14 recording spectrophotometer. Action spectra for fluorescence were determined using an Aminco-Keirs spectrophosphorimeter with an RCA 7102 photomultiplier tube and uncorrected for variation in spectral intensity of the Xenon lamp.

Results

Sedimentation Analysis. The sedimentation patterns of phycocyanin treated with PMPSA were examined at pH 7.0 (0.1 M phosphate buffer) and at pH 5.0 (0.1 M acetate buffer). After adding PMPSA to phycocyanin, the solutions were allowed to stand for 3 days at 4°. Each solution was subsequently treated with excess glutathione and also subjected to sedimentation analysis. The ultracentrifuge diagrams shown in Figures 1–4 exhibit a polydispersity due to a reversible equilibrium existing between the various forms of aggregate. Table I shows the results of the sedimentation analysis, $s_{20,w}$ values of these species having been obtained

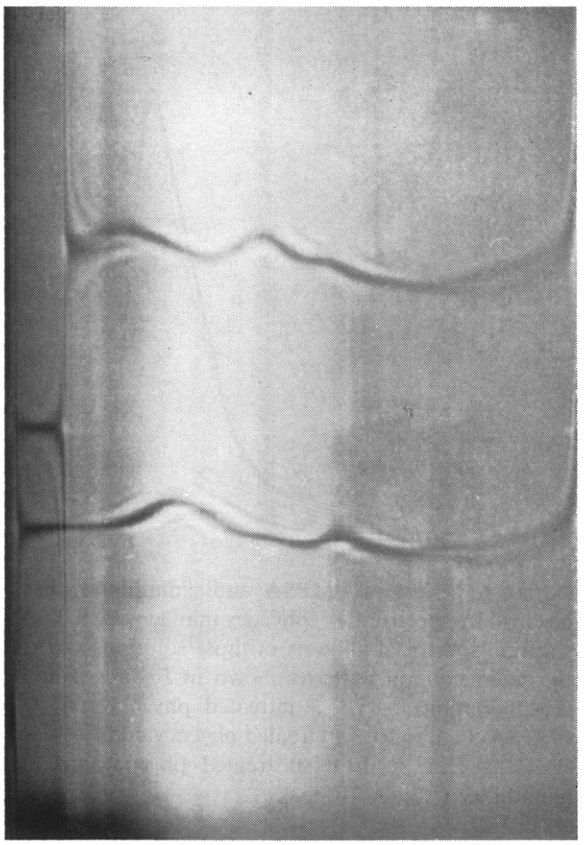

FIGURE 3: Effect of PMPSA (10^{-3} M) on the sedimentation pattern of phycocyanin (0.33%) at pH 5.0 (0.1 M acetate buffer, 1% NaCl). Lower sedimentation pattern: untreated phycocyanin; upper pattern: phycocyanin treated with PMPSA. Photographs were taken 27 min after the centrifuge reached 59,780 rpm. Menisci are on the left; temperature, 20°; phase-plate angle, 60°.

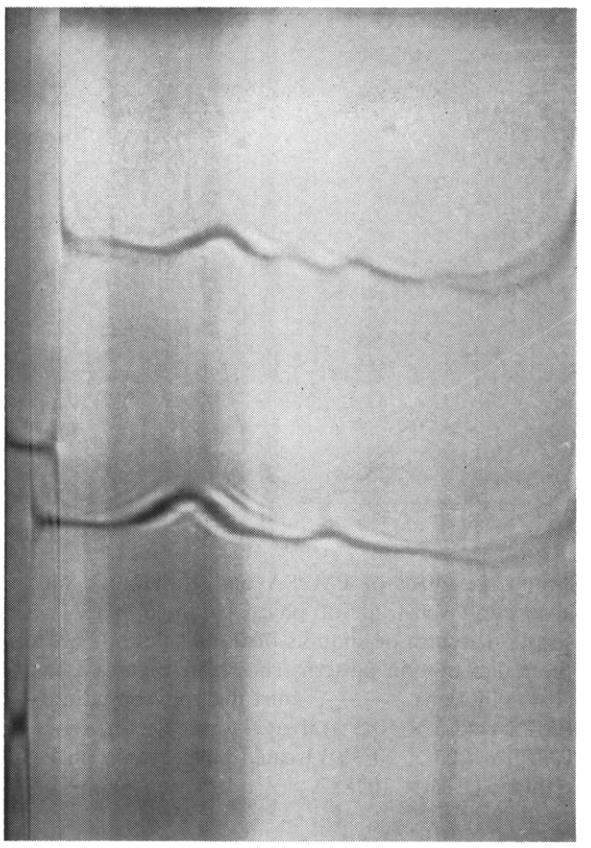

FIGURE 4: Effect of glutathione (5×10^{-3} M) on the sedimentation pattern of phycocyanin (0.33%) untreated and treated with PMPSA (10^{-3} M) at pH 5.0 (0.1 M acetate buffer, 1% NaCl). Lower sedimentation pattern: untreated phycocyanin plus glutathione; upper pattern: PMPSA-treated phycocyanin plus glutathione. Photographs were taken 25 min after the centrifuge reached 59,780 rpm. Menisci are on the left; temperature, 20°; phase-plate angle, 60°.

for each case. Those aggregates corresponding to the different $s_{20,w}$ values have been tentatively identified as monomer, trimer, hexamer, dodecamer, or higher aggregate as has been proposed by recent investigations (Berns *et al.*, 1964; Berns and Edwards, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965). Aggregates having $s_{20,w}$ values intermediate to those above have been assigned somewhere between the proposed forms.

Figure 1 shows the ultracentrifuge pattern of phycocyanin treated with 10^{-3} M PMPSA (upper pattern) as compared to that of untreated phycocyanin (lower pattern), at pH 7.0. Untreated phycocyanin at pH 7.0 contains a major 5.5S component as well as two minor components (10.2 and 16.4 S). These three components in the order of increasing $s_{20,w}$ value may correspond to the trimer, hexamer, and dodecamer, respectively. Our observation that the 5.5S component predominates at pH 7.0 is in good agreement with the findings made by other investigators (Hattori *et al.*, 1965; Scott and Berns, 1965). After treating the phycocyanin with PMPSA, only a single broad peak (5.0 S) was observed, possibly due to a heterogeneous mixture of subunits smaller than the trimer. The complete disappearance of the hexameric and dodecameric forms caused by the PMPSA was impressive. This splitting of the aggregates into smaller subunits will be further demonstrated later using Sephadex G-100 gel filtration.

Figure 2 depicts the effect of 5×10^{-3} M glutathione on the aforementioned treated and untreated phycocyanin at pH 7.0. The addition of glutathione to untreated phycocyanin has no effect (Figure 2, lower pattern), whereas phycocyanin treated with PMPSA definitely undergoes reversible change giving rise to those associated forms present in the original mixture (Figure 2, upper pattern). The original 5.5S, 9.3S, and 16.8S components are clearly regenerated. This reversible change will be further substantiated using Sephadex G-100 gel filtration.

The effect of PMPSA on phycocyanin at pH 5.0 is shown in Figure 3. Untreated phycocyanin at pH

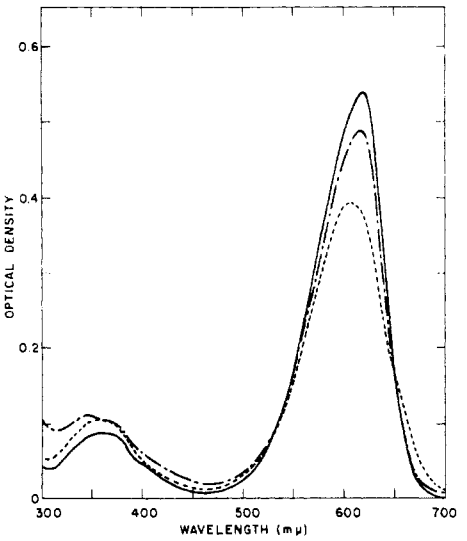

FIGURE 5: Effect of PMPSA and glutathione on the absorption spectrum of phycocyanin at pH 7.0. Absorption spectra of aliquots of those solutions used for the sedimentation patterns shown in Figures 1 and 2 (1:40 dilution). ———, untreated phycocyanin; ----, PMPSA- (2.5×10^{-5} M) treated phycocyanin; — - — -, PMPSA- (2.5×10^{-5} M) treated phycocyanin plus glutathione (1.25×10^{-4} M).

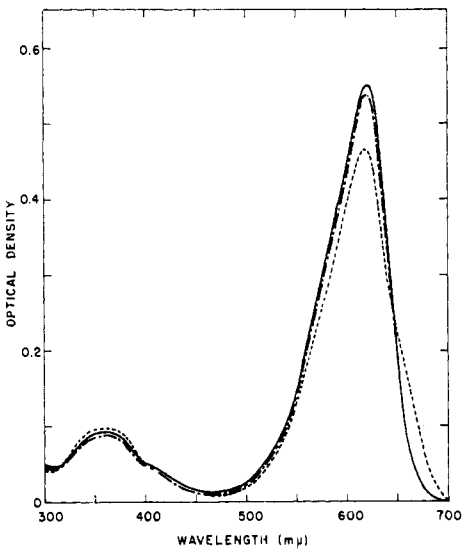

FIGURE 6: Effect of PMPSA and glutathione on the absorption spectrum of phycocyanin at pH 5.0. Absorption spectra of aliquots of those solutions used for the sedimentation patterns shown in Figures 3 and 4 (1:40 dilution). ———, untreated phycocyanin; ----, PMPSA- (2.5×10^{-5} M) treated phycocyanin; — - — -, PMPSA- (2.5×10^{-5} M) treated phycocyanin plus glutathione (1.25×10^{-4} M).

5.0 contains a major 9.2S component and a minor 17.1S component, presumably corresponding to the hexamer and dodecamer, respectively (Figure 3, lower pattern). The sedimentation coefficient of the hexamer, as we observed it, was slightly lower than those values (10–11 S) obtained by other investigators for phycocyanin from other species of red and blue-green algae (Eriksson-Quensel, 1938; Hattori and Fujita, 1959; Hattori *et al.*, 1965; Scott and Berns, 1965). In addition to these two components, we also observed an indication of two other small peaks, one falling between the 9.2S and 17.1S components and the other moving faster than the 17.1S component. This indicates the possible existence of other aggregates between hexamer and dodecamer as well as aggregates larger than the dodecamer. Previous investigations (Hattori *et al.*, 1965; Scott and Berns, 1965) have also shown that the greatest prevalence of the hexamer occurs at pH 5.0. Phycocyanin treated with 10^{-3} M PMPSA at pH 5.0 exhibits three peaks corresponding to $s_{20,w}$ values of 3.5, 10.1, and 11.7S (Figure 3, upper pattern). Since the 3.5S component has been shown to be the monomer (Berns *et al.*, 1964; Hattori *et al.*, 1965; Scott and Berns, 1965), it follows that it must have originated from some degree of dissociation of the hexamer or possibly aggregates larger than the hexamer which undergo incomplete dissociation into the 10.1S (hexamer) or 11.7S (intermediate aggregates somewhat larger than the hexamer) components. Thus it can be seen from Table I that phycocyanin is more completely dissociated by PMPSA into smaller subunits at pH 7.0 rather than at pH 5.0.

The addition of 5×10^{-3} M glutathione to phycocyanin treated with PMPSA at pH 5.0 demonstrates an interesting feature of its reversible change (Figure 4). Not only were the original 9.3S and 16.5S components regenerated but an intermediate 13.3S component as well as a heavy aggregate (~20S) were also clearly produced in amounts greater than originally present in untreated phycocyanin. This was not observed at pH 7.0. This increase in the amount of 13.3S and ~20S components presents the possibility of a rather random association among the smaller and intermediate subunits.

Absorption and Fluorescence. Other investigators (Bergeron, 1963; Goedheer and Birnie, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965) have recently observed a correlation between absorption characteristics and association states. Over the range pH 4–8, where dissociation of the major species from hexamer to trimer occurs, phycocyanin exhibits a decrease in absorbance of the 620-mμ maximum as well as a broadening of the band (Bergeron, 1963; Goedheer and Birnie, 1965). The same changes were observed by us and are shown in Figures 5 (pH 7.0) and 6 (pH 5.0). The difference between phycocyanin at pH 7.0 as opposed to pH 5.0 is better shown in fluorescence action spectra studies. The action spectrum of untreated phycocyanin at pH 7.0 exhibits two distinct maxima (Figure 7a) as shown previously (Berns *et al.*, 1963; Fujimori and

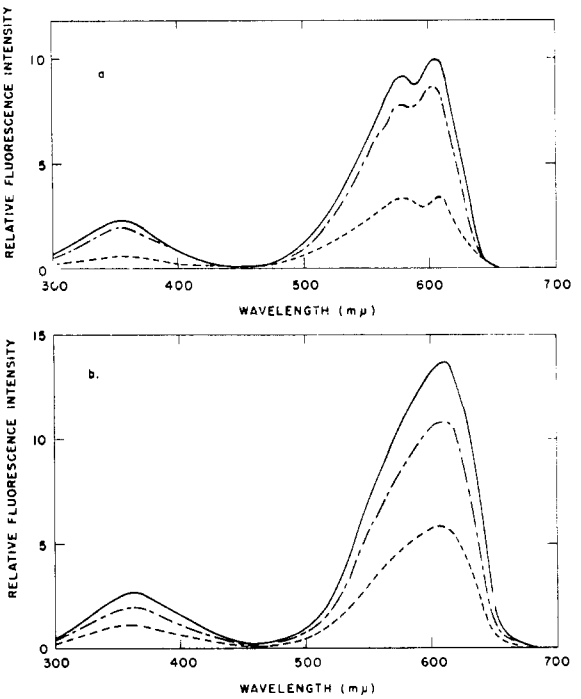

FIGURE 7: Effect of PMPSA and glutathione on the action spectrum of phycocyanin fluorescence at 645 mμ: (a) at pH 7.0 (0.1 M phosphate buffer) and (b) at pH 5.0 (0.1 M acetate buffer). ———, untreated phycocyanin; ----, PMPSA- (7.5×10^{-4} M) treated phycocyanin; — - — -, PMPSA- (7.5×10^{-4} M) treated phycocyanin plus glutathione (5×10^{-3} M).

Quinlan, 1963), whereas untreated phycocyanin at pH 5.0 (Figure 7b) was found to exhibit an apparently single band somewhat reminiscent of the absorption spectrum of Figure 6. The observation at pH 7.0 suggested that there exist two different pigment systems each absorbing at a different wavelength. The absorption maxima of each pigment system overlap with one another giving rise to a single absorption maximum. Changes in the proximity of the two bands, their relative intensities, and their capacity for fluorescence may account for the spectral differences observed at pH 7.0 and 5.0. The existence of two different pigment systems is further supported by studying the selective effect of sodium hydrosulfite on the fluorescence action spectra and absorption spectra of phycocyanin at pH 7.0. As the action spectrum of the 645-mμ fluorescence of Figure 8 shows, upon the addition of 0.01 M sodium hydrosulfite, the shorter wavelength peak disappears much sooner than the longer wavelength peak, also suggesting that the longer wavelength pigment is mainly responsible for this fluorescence.[3] Similar changes are observed in the absorption spectra as well.

[3] The possible presence of another fluorescence originating from the shorter wavelength pigment was pointed out in a previous paper (Fujimori and Quinlan, 1963).

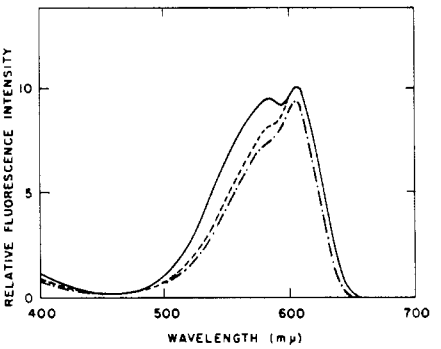

FIGURE 8: Effect of $Na_2S_2O_4$ (10^{-2} M) on the action spectrum of phycocyanin fluorescence at 645 mμ at pH 7.0 (0.1 M phosphate buffer). ———, before the addition of $Na_2S_2O_4$; -----, 6 min, and —·—·, 22 min after the addition of $Na_2S_2O_4$.

The decrease of the absorption maximum is accompanied by a shift to a longer wavelength.

Absorption spectra from aliquots of those solutions used for the sedimentation patterns shown in Figures 1–4 (1:40 dilution) are shown in Figures 5 (pH 7.0) and 6 (pH 5.0). The concentration of PMPSA after dilution is 2.5×10^{-5} M. The addition of PMPSA produced a decrease in absorption at the maximum which had now shifted to a shorter wavelength. This change was accompanied by a concomitant increase of absorption between 650 and 700 mμ. The decrease and shift of the peak were more pronounced at pH 7.0 than at pH 5.0. At pH 7.0, the original 620-mμ maximum shifted to 608 mμ and was reduced to about 73% of the initial value. At pH 5.0, the maximum was shifted to 617 mμ and was only reduced to 85% of the initial value. Increasing the concentration of PMPSA to 7.5×10^{-4} M at pH 7.0 caused a further decrease of absorption at the maximum which had now shifted to 605 mμ. The total decrease amounted to 55% of the initial value. The same increase in the concentration of PMPSA at pH 5.0 only brought about a slight further decrease in the absorption maximum which had now shifted to 615 mμ. As shown also in Figures 5 and 6, subsequent addition of glutathione causes a partial but definite regeneration of the heretofore diminished absorption as well as a disappearance of the increased absorption at 650–700 mμ.

These changes in absorption spectra were found to be correlated with those changes observed in fluorescence studies. The fluorescence action spectra of Figure 7 exhibit a marked decrease in fluorescence at both pH 7.0 and 5.0 in the presence of 7.5×10^{-4} M PMPSA. It should be noticed particularly at pH 7.0 that those changes occurring in the fluorescence action spectra are not the same as those taking place in the absorption spectra. In the former case, there is no shift of the peaks to shorter wavelengths, rather a uniform decrease in the entire spectrum. This indicates that the

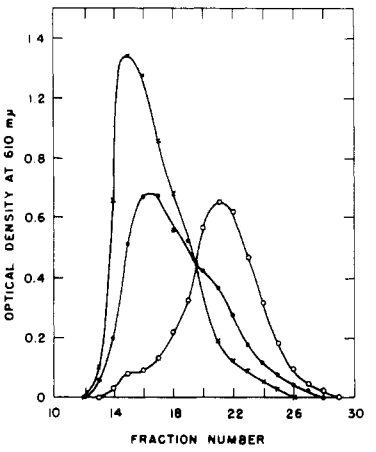

FIGURE 9: Sephadex G-100 gel filtration at pH 7.0. —×—×—, untreated phycocyanin, —○—○—, PMPSA- (10^{-2} M) treated phycocyanin; —●—●—, PMPSA- (10^{-2} M)- treated phycocyanin plus glutathione (1.5×10^{-2} M).

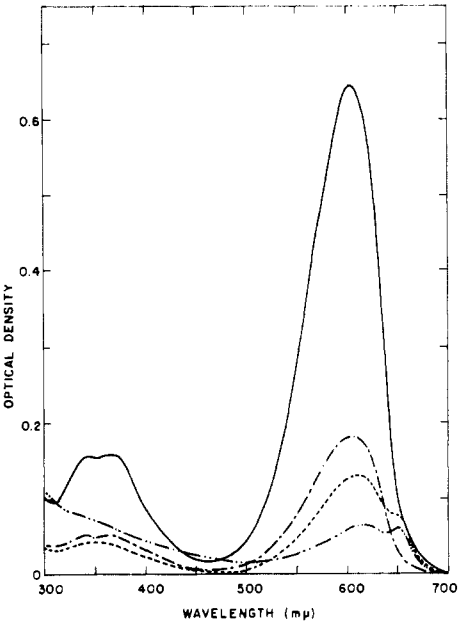

FIGURE 10: Absorption spectra of selected fractions from the gel filtration of PMPSA- (10^{-2} M) treated phycocyanin at pH 7.0. —··—··, fraction 15; -----, fraction 17; ———, fraction 21; —·—·, fraction 25.

645-mμ fluorescence originates mainly from the longer wavelength chromophore and that PMPSA affects this longer wavelength chromophore by decreasing its absorption and fluorescence. The shorter wavelength chromophore contributes to the fluorescence by transferring its excitation energy to the longer wavelength chromophore. This would account for the observed changes in absorption and fluorescence. The increase of absorption in the 650–700-mμ region will be discussed later in conjunction with the separation of the subunits. Partial regeneration of the fluorescence upon the addition of 5×10^{-3} M glutathione is also clearly established in Figure 7.

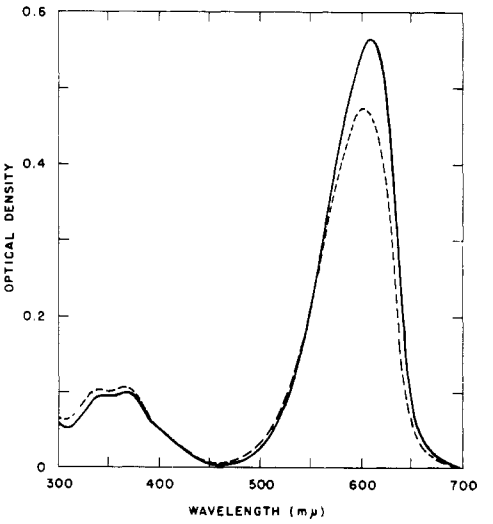

FIGURE 11: Effect of glutathione on the absorption spectrum of fraction 23 obtained from the gel filtration of PMPSA-treated phycocyanin at pH 7.0. -----, fraction 23; ———, fraction 23 plus 5×10^{-3} M glutathione.

Gel Filtration. Gel filtration using Sephadex G-100 was employed in order to effect a separation of the smaller subunits resulting from treating phycocyanin with PMPSA. We were unable to completely separate the several distinct aggregates of untreated phycocyanin at either pH 7.0 or 5.0. We, as well as other investigators (Scott and Berns, 1965), were able to observe that the leading fractions of phycocyanin at pH 7.0 emerging from the Sephadex G-100 column exhibited an absorption maximum at 625 mμ. The maximum gradually shifted to 610 mμ with the trailing fractions together with a gradual increase in the 620:350-nmμ optical density ratio. This phenomenon is indicative of incomplete separation of the heavy molecular weight units from the lighter.

After treating phycocyanin in this case with 10^{-2} M PMPSA at pH 7.0, we were able to successfully separate the smaller subunits using Sephadex G-100 gel filtration (Figure 9). Figure 10 shows the absorption spectra of fractions selected from the leading, middle, and trailing fractions. The small amount of heavier components appearing in the first few leading fractions revealed two separate absorption maxima at 615 and 652 mμ. Fractions containing the smaller subunits exhibited an absorption maximum at 605 mμ with two distinctly separated bands at 367 and 342 mμ. These observations indicate that the shift of the absorption maximum of phycocyanin, when treated with PMPSA, along with the increase in absorption between 650 and 700 mμ, is attributable to two separate spectral changes occurring in distinctly different subunits.

Glutathione was added to the separated fractions containing the heavier components and no spectral changes were observed. Figure 11 shows the increase in absorption and the shift to a longer wavelength upon addition of glutathione to a separated fraction containing smaller components. This indicates that the two pigment systems are present in the smaller subunits. Of the two, only the longest wavelength chromophore is effectively modified by PMPSA. Figure 9 clearly depicts the reassociation of the dissociated subunits upon addition of glutathione. As shown in Figure 5, this reassociation caused the increased absorption at 650–700 mμ to disappear.

The situation at pH 5.0 is not as simple as at pH 7.0 where most of the larger aggregates are dissociated into smaller subunits. We observed that the leading fractions from gel filtration of untreated phycocyanin at pH 5.0 exhibited absorption maxima at 625 mμ shifting gradually to 615 mμ with subsequent fractions. This shift to the shorter wavelength was not quite as pronounced as that observed at pH 7.0. Treatment of phycocyanin at pH 5.0 with 10^{-2} M PMPSA still gave rise to major quantities of aggregates, which are somewhat bigger than the hexamer, with a concomitant disappearance of the dodecamer and higher aggregates. These aggregates moved faster on the Sephadex column than did the hexamer present in untreated phycocyanin, but we were unable to effect a clean-cut separation of these aggregates from the smaller subunits. Most of the leading fractions exhibited an absorption maximum at 615 mμ whereas the trailing fractions showed the maximum shifted to 610 mμ.

Discussion

PMPSA effectively induces dissociation of phycocyanin and the dissociated molecules are able to undergo association upon the subsequent addition of glutathione. The extent of this dissociation–association is pH dependent. At pH 5.0, near the isoelectric point (pH 4.4–4.7) of phycocyanin (Svedberg and Katsurai, 1929; Hattori and Fujita, 1959), the predominant hexamer and other higher aggregates undergo incomplete dissociation giving rise to species in varying stages of disaggregation. Upon addition of glutathione, the random reassociation of these newly dissociated species gives rise to the initially present forms, differing only in relative amounts of each. At pH 7.0, where the net charge on the protein is increased, the predominant trimer and other higher aggregates are all converted to subunits smaller than the trimer. The addition of glutathione regenerates the trimer, hexamer, and dodecamer in the same ratio as the original associated forms, indicating an orderly transition.

If all of the aggregates are composed of the same smallest subunit, as has been postulated, one can expect that complete dissociation will give rise to only one kind of basic subunit. Our ability to separate the dissociated subunits by gel filtration at pH 7.0 reveals that the subunits of phycocyanin from *A. nidulans* are not all identical, and that phycocyanin consists of two different kinds of subunit, one characterized by the 605-mμ absorption band (the major subunit), the other by the 615- and 652-mμ bands (the minor subunit). The minor subunit is present in small amounts and therefore would not be expected to severely alter any of the basic structures postulated as being composed of monomeric subunits, but knowledge of the precise structure awaits further investigation.

It is known that sulfhydryl groups play some role in the association of subunits for many proteins. The ability of *p*-mercuribenzoate to disaggregate the enzymes of muscle phosphorylase (Madsen and Cori, 1956), yeast alcohol dehydrogenase (Snodgrass *et al.*, 1960), and aspartate transcarbamylase (Gerhart and Schachman, 1965) has been demonstrated. Other proteins such as hemerythrin (Keresztes and Klotz, 1963) and hemoglobin (Bucci and Fronticelli, 1965) undergo a similar dissociation. The role of sulfhydryl groups in hydrophobic interactions within proteins has recently been suggested (Godschalk and Veldstra, 1965; Cecil and Thomas, 1965). The hydrophobic interaction is discussed as one of the probable forces involved in the specific protein–protein interactions in phycocyanin (Scott and Berns, 1965). It has also been suggested that the nature of the environment surrounding the pigment moieties in phycocyanin is probably hydrophobic (Ó hEocha, 1963).

Phycocyanin from the blue-green algae *Microcystis aeruginosa* and *Nostoc muscorum* contains phycocyanobilin as the prosthetic group (Ó hEocha, 1963). A previous investigation (Fujimori and Quinlan, 1963) suggested that there are two types of pigment systems in phycocyanin from *A. nidulans* each absorbing at a different wavelength. This has also been shown for phycocyanin from other species of algae (Ó hEocha, 1965). This present investigation reveals the presence of these two pigment systems in the major subunit of phycocyanin. The existence of a third pigment system absorbing at 615 and 652 mμ has also been observed in the minor subunit.

Not only does PMPSA induce dissociation of the protein, but it also gives rise to a change in the absorption and fluorescence characteristics of the pigment moieties present in the molecule. The observed change may well be attributed to the dissociation of the protein molecule into smaller subunits producing an environmental change in the pigment vicinity especially since these changes are most prominent at pH 7.0 where dissociation is almost complete. The three pigment systems mentioned above seem to behave differently to the conformational change induced by PMPSA. The pigment moiety in one of the pigment systems of the major subunit appears to undergo a specific interaction in that protein environment where the conformation is particularly sensitive to the sulfhydryl groups and hence the degree of association. This interaction gives rise to an enhancement of absorption accompanied by a shift to longer wavelengths and also brings about an increase in fluorescence. Whether the difference in absorption characteristics of these three pigment systems is due to nonidentical types of pigment or to

the same pigment (phycocyanobilin) only located or oriented differently in the protein environment is not clear at this time.[4]

It would thus appear that sulfhydryl groups play an important role in the association of the subunits comprising the phycocyanin molecule, in the shift of pigment absorption to longer wavelengths, and in the enhancement of absorption and fluorescence. These factors are considered to be favorable for efficiently transferring energy from phycocyanin to chlorophyll during photosynthesis. It has been proposed (Scott and Berns, 1965) that the most efficient optimal structure for energy transfer to the chlorophyll molecule is when phycocyanin is present as the hexamer.

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References

Bergeron, J. A. (1963), *in* Photosynthetic Mechanisms of Green Plants, Washington, D. C., National Academy of Sciences, National Research Council, p 527.

Berns, D. S., Crespi, H. L., and Katz, J. J. (1963), *J. Am. Chem. Soc. 85*, 8.

Berns, D. S., and Edwards, M. R. (1965), *Arch. Biochem. Biophys. 110*, 511.

Berns, D. S., and Scott, E. (1966), *Biochemistry 5*, 1528.

Berns, D. S., Scott, E., and O'Reilly, K. T. (1964), *Science 145*, 1054.

Bucci, E., and Fronticelli, C. (1965), *J. Biol. Chem. 240*, 55.

Cecil, R., and Thomas, M. A. W. (1965), *Nature 206*, 1317.

Eriksson-Quensel, I. B. (1938), *Biochem. J. 32*, 585.

Fujimori, E. (1964), *Nature 204*, 1091.

Fujimori, E., and Quinlan, K. (1963), *in* Photosynthetic Mechanisms of Green Plants, Washington, D. C., National Academy of Sciences, National Research Council, p 519.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Godschalk, W., and Veldstra, H. (1965), *Arch. Biochem. Biophys. 111*, 161.

Goedheer, J. C., and Birnie, F. (1965), *Biochim. Biophys. Acta 94*, 579.

Hattori, A., Crespi, H. L., and Katz, J. J. (1965), *Biochemistry 4*, 1225.

Hattori, A., and Fujita, Y. (1959), *J. Biochem. (Tokyo) 46*, 633.

Keresztes, S., and Klotz, I. M. (1963), *Biochemistry 2*, 923.

Madsen, N. B., and Cori, F. (1956), *J. Biol. Chem. 223*, 1055.

Ó hEocha, C. (1963), *Biochemistry 2*, 375.

Ó hEocha, C. (1965), *Ann. Rev. Plant. Physiol. 16*, 415.

Scott, E., and Berns, D. S. (1965), *Biochemistry 4*, 2597.

Snodgrass, P. J., Vallee, B. L., and Hoch, F. L. (1960), *J. Biol. Chem. 235*, 504.

Svedberg, T., and Eriksson, I. B. (1932), *J. Am. Chem. Soc. 54*, 3998.

Svedberg, T., and Katsurai, T. (1929), *J. Am. Chem. Soc. 51*, 3573.

Svedberg, T., and Lewis, N. B. J. (1928), *J. Am. Chem. Soc. 50*, 528.

[4] Similar, but more pronounced, phenomena have recently been observed in phycoerythrin (E. Fujimori and J. Pecci, 1966, unpublished data).