

## Properties and Ultrastructure of Phycoerythrin From *Porphyridium cruentum*<sup>1, 2</sup>

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Received June 23, 1969.

**Abstract.** Phycoerythrin, a photosynthetic accessory pigment, was isolated from *Porphyridium cruentum* and examined by electron microscopy and disc gel electrophoresis. The absorption monomer, with maxima at 563, 545, and a shoulder at 500 nm, has a molecular weight of about 300,000. With phosphotungstic acid staining it appears as a tightly structured disc-shaped particle possessing a mean diameter of  $101 \pm 0.4\text{\AA}$  and height of  $54 \pm 0.7\text{\AA}$ . The absorption maxima remained the same in glutaraldehyde fixed material, and in dimer and trimer aggregates. Treatment with sodium dodecyl sulfate caused a breakdown into smaller units accompanied by a loss of the 563 nm peak. It is suggested that this absorption monomer is the *in vivo* functional species and comparable to the phycocyanin hexamer, but structurally distinguishable at the ultrastructural level. It has been calculated that about 35 phycobiliprotein molecules can be contained within each phycobilisome. There are  $1.4 \times 10^3$  chlorophyll molecules per phycobilisome, but not contained within it.

Our previous work (10) has shown that the photosynthetic accessory pigments in *Porphyridium cruentum* are located on the lamellae in the form of phycobilisomes (*in vivo* phycobilin aggregates). Absorption spectra of isolated phycobilisomes indicate that phycoerythrin as well as phycocyanin are present. Because only 1 type of phycobilisome is present in *P. cruentum*, and since phycocyanin is a necessary intermediate in the effective energy transfer between phycoerythrin and chlorophyll (6, 8) the 2 phycobilins must be very closely associated. Both pigments are believed to be within each individual aggregate.

To understand the *in vivo* relationship of the 2 phycobiliproteins they must be distinguished at the ultrastructural level. Therefore, phycoerythrin was isolated from the red alga *P. cruentum* to study its structure in order to be able to compare it with phycocyanin. The work of Berns and Edwards (2) indicates that the purified C-phycocyanin, isolated from *Plectonema calothricoides*, in its most stable form exists as a hexameric ring with a central hole, an overall diameter of 130Å and a molecular weight of about 200,000. It remains to be shown whether or not phycoerythrin has a similar shape and appearance as the phycocyanin hexamer, and in what way, if any, it differs. To permit a meaningful comparison of the 2 phycobilins in the present work,

preparations of phycoerythrin were monitored by electron microscopy, absorption spectrophotometry, and the molecular weight of the smallest stable particle was determined.

### Materials and Methods

Phycoerythrin was obtained from *Porphyridium cruentum* which had been grown in axenic cultures in an artificial sea water medium (14) at room temperature. The liquid cultures, aerated with 1% carbon dioxide and 99% air, were continuously agitated and illuminated with cool white fluorescent lamps (incident intensity 350 ft-c).

Cells were harvested by centrifugation (10,000g) and rinsed twice in 0.01 M phosphate buffer (sodium and potassium) pH 6.8. The pellets were frozen in polyethylene tubes in an acetone-dry ice bath and stored in a freezer until used. For use the pellets were thawed and the slurry of cells was passed several times through a No. 20 syringe needle in order to break the cell clumps prior to breaking in an Aminco-French pressure cell at 8 to 12,000 psi.

The phycoerythrin isolation method, involving *n*-butanol and ammonium sulfate fractionation, described by Leibo and Jones (15) was followed in most essentials. Throughout the entire isolation the preparation was kept at about 4°. For the precipitations a solution of supersaturated ammonium sulfate was used in decreasing concentrations (35-20% of saturation) and repeated until a 545/275 nm absorbance ratio of 5 or higher was obtained. Final crystallization of phycoerythrin was accomplished by addition of recrystallized ammonium sulfate powder (15-20% of saturation).

<sup>1</sup> Research was supported by the United States Atomic Energy Commission Contract No. AT(30-1)3913, and the Smithsonian Institution.

<sup>2</sup> Published with the approval of the Secretary of the Smithsonian Institution.

The criteria of purity were the following: a) a 545/275 nm absorbance ratio of 5 or higher, b) homogeneity on polyacrylamide gel (see results), and c) uniformity of structure when examined by electron microscopy.

**Electron Microscopy.** Phycoerythrin crystals were dissolved in 0.001 M phosphate buffer pH 6.0, 6.8, and 7.0 and dialyzed against the same buffer, then centrifuged for 30 min at 100,000*g*. It was necessary to use low ionic strength phosphate buffer in order to prevent obliteration of detail when the material was negatively stained. For glutaraldehyde fixation the ammonium sulfate was removed by dialysis against 0.01 M phosphate (pH 6.8 generally), then 2% or 4% buffered glutaraldehyde was added directly and allowed to react for 1 hr, or it was allowed to enter through the dialysis membrane overnight. The negative stains were always prepared shortly before use. The 2% solutions of phosphotungstic acid (PTA) were adjusted to the appropriate pH (usually 6.8) with ammonium hydroxide, while uranyl oxalate was prepared according to the procedure of Mellema *et al.* (17). Other negative stains such as potassium tungstate, sodium tungstate, sodium silicotungstate, and uranyl acetate were also tried. However, because of inconsistent results or precipitation of the protein (with uranyl acetate) they were not normally used. Phosphotungstic acid at pH 6.8 was commonly employed because it yielded the most consistent results and because it made more valid comparisons with other phycobiliproteins possible (2, 5).

Phycoerythrin was diluted with buffer and further by phosphotungstic acid or uranyl oxalate to a final concentration of 5 to 25  $\mu\text{g}/\text{ml}$  and applied to formvar carbon-coated grids which had been ionized with a 5000 volt glow discharge in a vacuum evaporator, to enhance spreading. Carbon-coated "holey" formvar nets were tried and discontinued after observing that distortions in shape and size of the phycoerythrin particles occurred. Control grids were prepared by applying the buffer plus PTA, but no reproducible patterns could be seen which corresponded to the phycoerythrin preparations. The grids were studied in a Philips EM 200 electron microscope at 60 or 80 kv with double condenser illumination and liquid nitrogen specimen cooling. Records were made on 35 mm fine grain positive film (Eastman Kodak No. FRP 426) at a magnification of 33,200. Measurements of the particles were made from photographically enlarged areas of preparations such as Fig. 1. Only those particles were measured which had the largest diameters as well as the most distinct boundaries. This was justified by assuming that those more clearly visible were least buried in the stain, and since we dealt with a homogenous population there was little possibility of selecting for one particular species. This usually was not a problem since with very thin spreading it was possible to measure almost every particle.

**Disc Gel Electrophoresis.** For the molecular weight determination of phycoerythrin the acrylamide gel electrophoresis method of Hedrick and Smith (13) was followed with 1 minor modification. It was necessary to reduce the recommended concentration of the ammonium persulfate catalyst to prevent too rapid gelation and release of heat. The ammonium persulfate concentration was 0.047g/100 ml for 4% and 5% gel, and 0.028g/ml for 6 to 8% gel. The final pH of the small pore solution was pH 7.9, and the large pore solution pH 5.7, while the reservoir buffer (0.034 M asparagine neutralized with Tris) was pH 7.3. The commercially available Buchler Polyanalyst (Buchler Instruments, Incorporated, Fort Lee, New Jersey) apparatus was used.

Small pore gel concentrations ranged in 1% increments from 4 to 8%. The phycoerythrin was diluted in a 50% glycerol solution, and layered between the spacer gel and reservoir buffer. Each tube received about 25  $\mu\text{g}$  of protein. Electrophoresis was at room temperature (2 mA per tube initially and 4 mA after the dye front passed into the small pore gel). After putting the gels into ice water, to facilitate their intact removal from the tube, the dye front was marked by insertion of a thin piece of nichrome wire. All visible bands were measured before and after fixing in trichloroacetic acid and staining with Coomassie blue or amido schwarz.

To verify the reliability of the method in our hands, several proteins of known molecular weight were used as reference markers: bovine serum albumin (Nutritional Biochemical Corporation, Cleveland, Ohio), catalase (CalBiochem, Los Angeles, California), alpha-amylase (Sigma Chemical Company, St. Louis, Missouri), and ferritin (Mann Research Laboratories, New York, New York).

For the recovery of the phycoerythrin bands from the gels the same procedure was followed except the protein concentration was increased to 50 to 75  $\mu\text{g}$  per tube. Advantage was taken of the fact that glutaraldehyde stabilizes the phycobiliprotein aggregates, which then allows their recovery in greater concentration than in the unfixed material. Best results were obtained with phycoerythrin which had been fixed in glutaraldehyde and then stored in 20% ammonium sulfate (once fixed, phycoerythrin does not crystallize again in ammonium sulfate). Of the several distinct pink bands which formed on the gel, the 3 which had migrated the farthest, were cut out with a razor blade. The excess gel was removed and the separate pink bands were collected in 5 ml tubes and macerated with a close fitting stirring rod. Twelve or 24 gel slices were used for each extraction. To extract the pigment about 2 ml of cold buffer or 2% PTA (pH 6.8) was added to each tube. This gel slurry was then transferred to a larger centrifuge tube and agitated on a magnetic stirrer for 1 to 2 hr then centrifuged at top speed in an International clinical centrifuge. The clear

pink supernatant solutions were used for spectrophotometric readings (on a Cary 14 spectrophotometer) and/or preparation of electron microscope grids within 30 to 120 min after extraction. It was necessary to use the material as soon as possible because some dissociation occurred on storage.

**Sodium Dodecyl Sulfate Treatment.** Phycoerythrin in 0.01 M sodium phosphate buffer pH 6.8 and 1% sodium dodecyl sulfate (SDS) was incubated for 3 hr at 37° and dialyzed overnight against the same buffer concentration but with a reduction to 0.1% SDS. The control preparations were exposed to the same conditions with the omission of SDS. At the termination of the incubation the SDS-treated pigment had changed in color from pink to purple. The treated material did not have the usual bright orange fluorescence noticeable in white light.

## Results

**Structure of Phycoerythrin.** Phycoerythrin is one of the most stable chromoproteins and its isolation from *P. cruentum* and purification were readily accomplished. It is known to be stable over a wide range of pH (3–10) (19,20) and the present conditions were well within that limit. A pure preparation characterized by a 545/275 nm absorbance ratio of 5 or higher displayed one major pink band on polyacrylamide gel. Sometimes a faint second and suggestion of a third band were present. After staining for protein, the only visible bands were those corresponding to the original pink phycoerythrin bands.

A preparation of phycoerythrin when negatively stained with uranyl oxalate, is seen to consist primarily of monomers (see Discussion for definition). In Fig. 1 various aspects of the phycoerythrin particles are evident. Those with a rounded appearance are believed to be views of the flat side of the particles which are envisioned as being disc shaped with an axial ratio of 2:1. Since there is a tendency for the particles to dry on the grid with their flat side up, views of their narrow side are less common. Side views are more easily found where there is a build up of stain and pigment, and of course in the case of linear aggregates (Fig. 3). The first 2 enlargements (a and b) in Fig. 2 are of the broad face view of phycoerythrin particles, and c and d are side views.

Uranyl oxalate staining resulted in pictures with greater detail. However, with either stain the particles appear very tightly structured. Some substructure is evident in Figs. 1 and 2, although one cannot detect a definite subunit shape nor any regular array of the substructure. There were no structural differences among preparations at pH 6.0, 6.8, and 7.0.

Measurements were made of the particle width and height on uranyl oxalate and PTA stained preparations. With uranyl oxalate staining the mean

diameter of the broad face view was  $115 \pm 0.8\text{\AA}$  (SE). This also is the approximate diameter of the long aspects of the side views. The mean particle height (the short axis) measured  $64 \pm 0.6\text{\AA}$ . With phosphotungstic acid staining the mean diameter of the rounded face view is  $101 \pm 0.4\text{\AA}$  and the height is  $54 \pm 0.7\text{\AA}$ . A discrepancy of about  $14\text{\AA}$  is readily evident in the values obtained from the 2 differently stained preparations, however, as is pointed out in the Discussion the values obtained from the PTA preparations are believed to be more reliable.

**Properties of Phycoerythrin.** A typical absorption spectrum of phycoerythrin from *P. cruentum* shown in Fig. 6 characteristically has a shoulder at 500 nm and peaks at 545 and 563 nm. Treatment with glutaraldehyde stabilizes the phycoerythrin aggregates but causes no noticeable shift in the absorption peaks. However, the bright orange fluorescence noted under white light is diminished upon glutaraldehyde fixation.

A preparation fixed in glutaraldehyde contains aggregates of various sizes and shapes (Fig. 3). A mixture of linear and globular aggregates is seen. There appears to be considerable freedom of choice in the manner of aggregation. The linear figures are formed by association of the particles along their broad faces. However, they can also associate along their narrow faces, or along the narrow of one and broad face of another. This allows formation of 3 dimensional configurations such as the phycobilisomes.

When a glutaraldehyde-fixed phycoerythrin preparation (Fig. 3) is subjected to discontinuous electrophoresis on polyacrylamide gel several distinct bands result (Inset Fig. 7). As in the untreated phycoerythrin, all the material visible on the stained gel corresponded to the phycoerythrin bands prior to staining. The 3 bands which migrated fastest were extracted and studied. The first band, nearest the front, when examined by electron microscopy consisted entirely of monomers. Dimers (Fig. 4) were the predominant species in the second band, and trimers (Fig. 5) in the third band. Some monomers were found in the negative stained preparations from band II, while dimers and monomers were present in preparations from band III. This can either be due to incomplete separation on the gel, or what is more likely, the larger aggregates are more readily dissociated by handling and drying on the electron microscope grids.

Of the 3 bands examined, the monomer band was always the most dense and had a bright orange fluorescence. As the aggregates increased in size, the intensity of the bands decreased. Monitoring the extracts from the gel bands revealed (Fig. 7) that the absorption maxima are the same in the monomer, dimer, and trimer preparations. Therefore, aggregation of phycoerythrin particles at this level does not cause a shift in the characteristic

absorption peaks of phycoerythrin (563, 545, and a shoulder at 500 nm).

**Molecular Weight Determination.** The method of Hedrick and Smith (13) was used for the molecular weight determination of phycoerythrin. By this method, using disc gel electrophoresis, size isomers can be distinguished from charge isomers. The mobility of the 3 fastest moving bands of phycoerythrin was followed on 4 to 8% gel. When the log of the phycoerythrin mobility relative to the dye front was plotted against gel concentration, a group of non-parallel lines intersecting around 0% gel concentration resulted (Fig. 8). This indicates that these are size isomers, *i.e.* the aggregates separated according to size.

To determine the reliability of this technique under our conditions, proteins of known molecular weight were run on disc gels. Then the slopes from plots of the log relative mobility *versus* gel concentration were determined. When the negative slopes of the chosen proteins (alpha-amylase 45,000, bovine serum albumin monomer 65,000, catalase 240,000, and ferritin monomer 450,000) were plotted against molecular weight (Fig. 9) there was very good agreement between our results and those of Hedrick and Smith (13). The plot of Fig. 9 was taken as the standard. By plotting the negative slope of 12.5, the phycoerythrin monomer comes very near catalase and has a molecular weight of about 270,000 while the dimer (20.5) is close to ferritin and has a relative molecular weight of about 500,000. The monomer molecular weight value is considered to be most reliable because the dimer is near the limit of resolution. The trimer had to be ignored, since this system's accuracy is limited to the molecular weight range of 50,000 to 500,000.

Exposure of phycoerythrin to 1% sodium dodecyl sulfate for 3 hr at 37° resulted in the loss of fluorescence and a shift in color from the usual pink to purple. The absorption spectrum of such a preparation showed that the 563 nm peak was absent. When subjected to electrophoresis on 5% polyacrylamide gel a fast moving purple band appeared followed by a more slowly moving pink band, which contained the monomers. Examination by electron microscopy of the extracted purple material revealed that only very small units were present which were difficult to distinguish from the background. It was, therefore, concluded that sodium dodecyl sulfate caused a breakdown of the phycoerythrin monomer, and that due to this breakdown there was a change in the color.

### Discussion

The smallest stable phycoerythrin particle possessing absorption maxima at 563, 545, and a shoulder at 500 nm with an approximate molecular weight of 300,000 is considered a monomer. This is the "native" (15) B-phycoerythrin molecule (for no-

menclature designation see 20, 22), and probably the *in vivo* functional species. Supporting reasons are: A) Phycoerythrin is a stable chromoprotein which when isolated, normally has a molecular weight of about 200,000-300,000, and B) has absorption maxima of 563, 545, and 500 nm, corresponding closely to *in vivo* absorption, C) when this particle is broken down into smaller units a change in the absorption results, while upon aggregation there is no absorption change.

Most reported phycoerythrin molecular weights are in the 200,000-300,000 range (1, 3, 7, 19, 20, 22). Therefore, the value of 270,000 obtained here by disc-gel electrophoresis and the 340,000 calculated from the electron micrograph measurements are in reasonable agreement.

This particle is by no means the smallest subunit obtainable, as shown for instance by the sodium dodecyl sulfate treatment, but it is the smallest particle which has the typical absorption maxima. Since absorption spectra of various smaller fractions are often not reported, it is difficult to judge if all manifest an absorption change. Neufeld (19) identified a purple component, which lacked the 563 nm peak, as a subunit with a molecular weight of 40,000. Mieras and Wall (18) who recently reported molecular weights of 23,000, 36,000 and 85,000 for B-phycoerythrin also mentioned a purple component, which implies the loss of the 563 nm peak. Fujimori and Pecci (9) found that one of the subunits created by *p*-chloromercuribenzoate treatment lacked the same peak. They believed that they were dealing with a subunit which had only one of the 2 phycoerythrin chromophore types.

If the various results are considered in terms of the existence of one physiologically active phycoerythrin chromophore (phycoerythrobilin), demonstrated by Chapman *et al.* (4), one is led to assume that the changes are due to alterations of the protein environment to which the phycoerythrobilins are attached.

A diameter of 101Å and particle height of 54Å with phosphotungstic acid are believed to be the true values, not those of 115Å by 64Å derived from uranyl oxalate stained material. The molecular weight calculations, using a radius of 50.5Å and height of 54Å with an assumed density of 1.3, give a value of 340,000 which is close to that derived by other methods (1, 3, 19). Using a radius of 57.5Å and height of 64Å, the calculated molecular weight is 530,000 about 2 times as high. Uranyl oxalate gives an excellent image as Mellema *et al.* (17) claim. However, it appears that a swelling is caused by this stain which could account for the larger size as well as for greater detail in the phycoerythrin particle (compare Figs. 1 and 3). It should be noted that very interesting patterns can be obtained by the photographic rotation enhancement technique (16) showing 5, 6, or more distinct units per particle. They were not held to be meaningful because it was not possible to observe these patterns directly

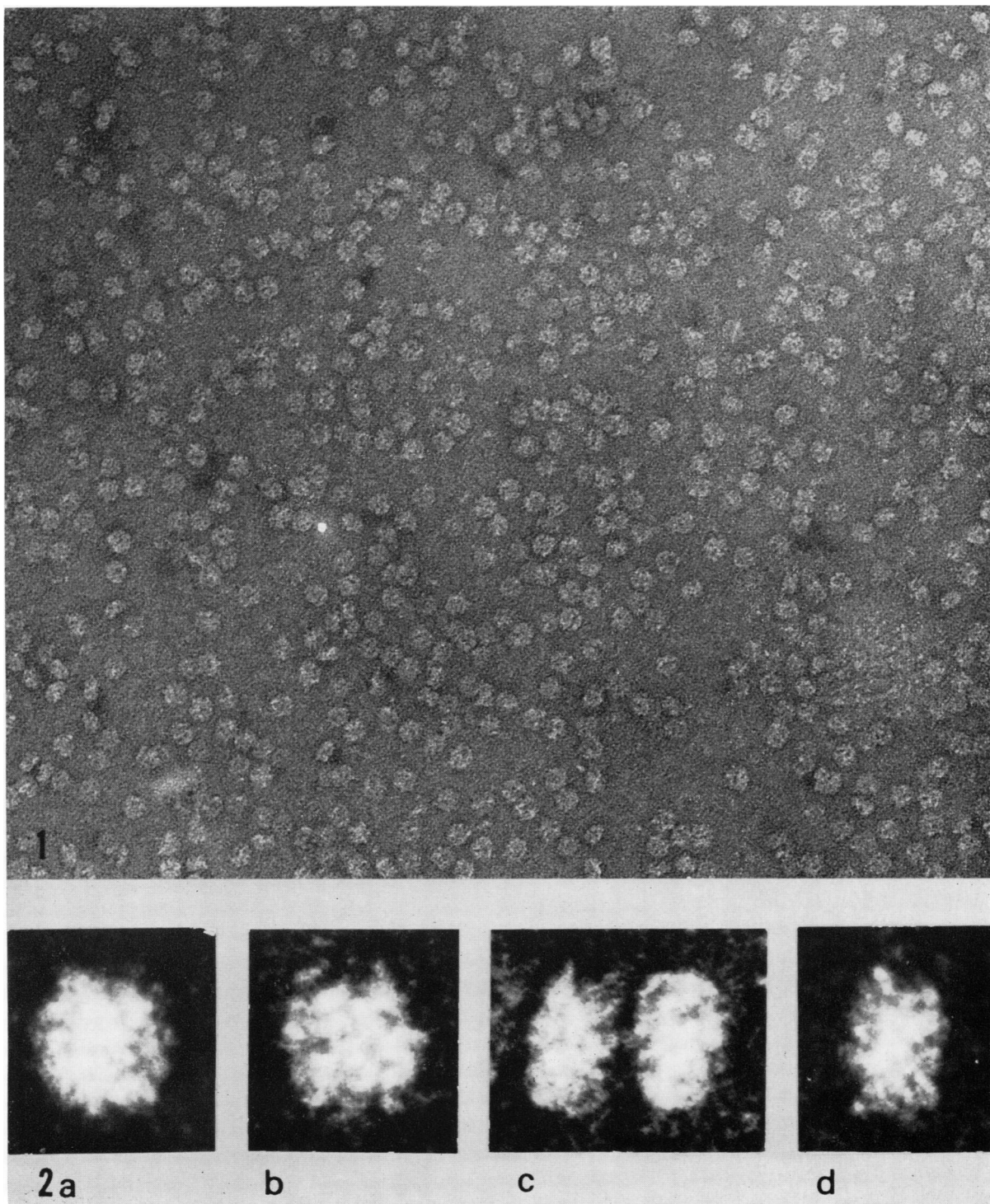


FIG. 1. An electron micrograph of phycoerythrin stained with uranyl oxalate. Most of the particles are oriented with their broad faces in full view. Side views are also present but less common. Substructure can be noted, however, individual subunits cannot be discerned.  $302,000\times$ .

FIG. 2. Enlargements of phycoerythrin particles stained with uranyl oxalate. Two broad face views of the disc shaped particles are seen in a and b, and side views in c and d. All are at the same magnification.  $2,100,000\times$ .

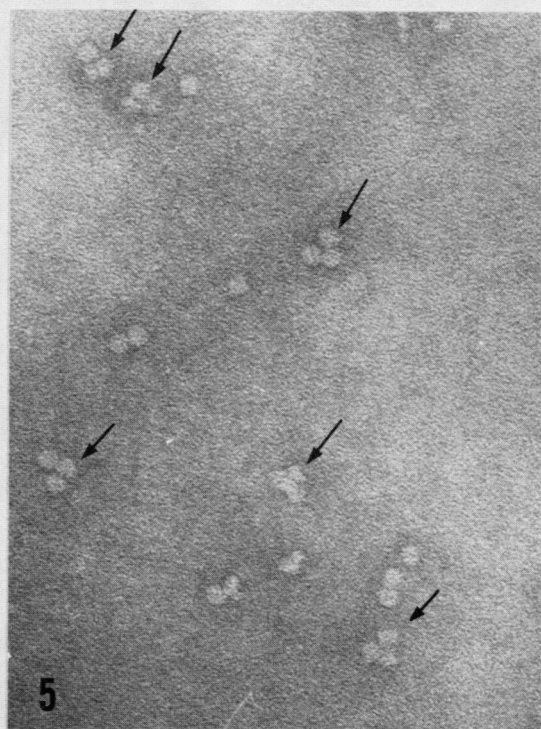
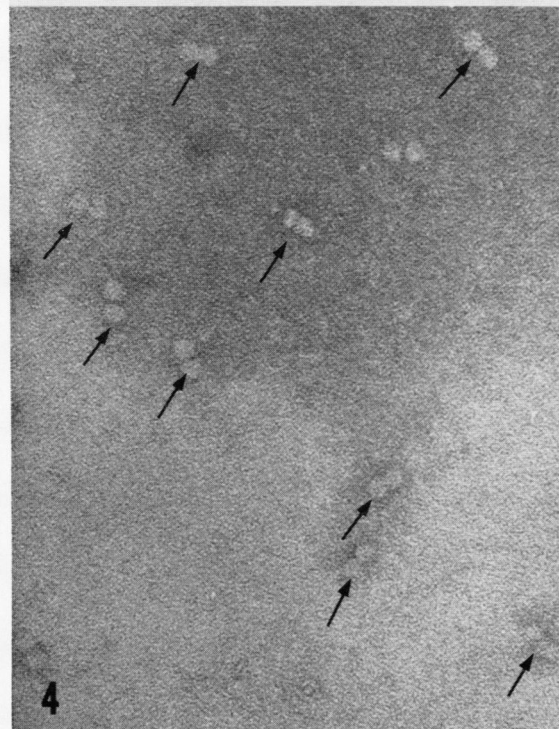
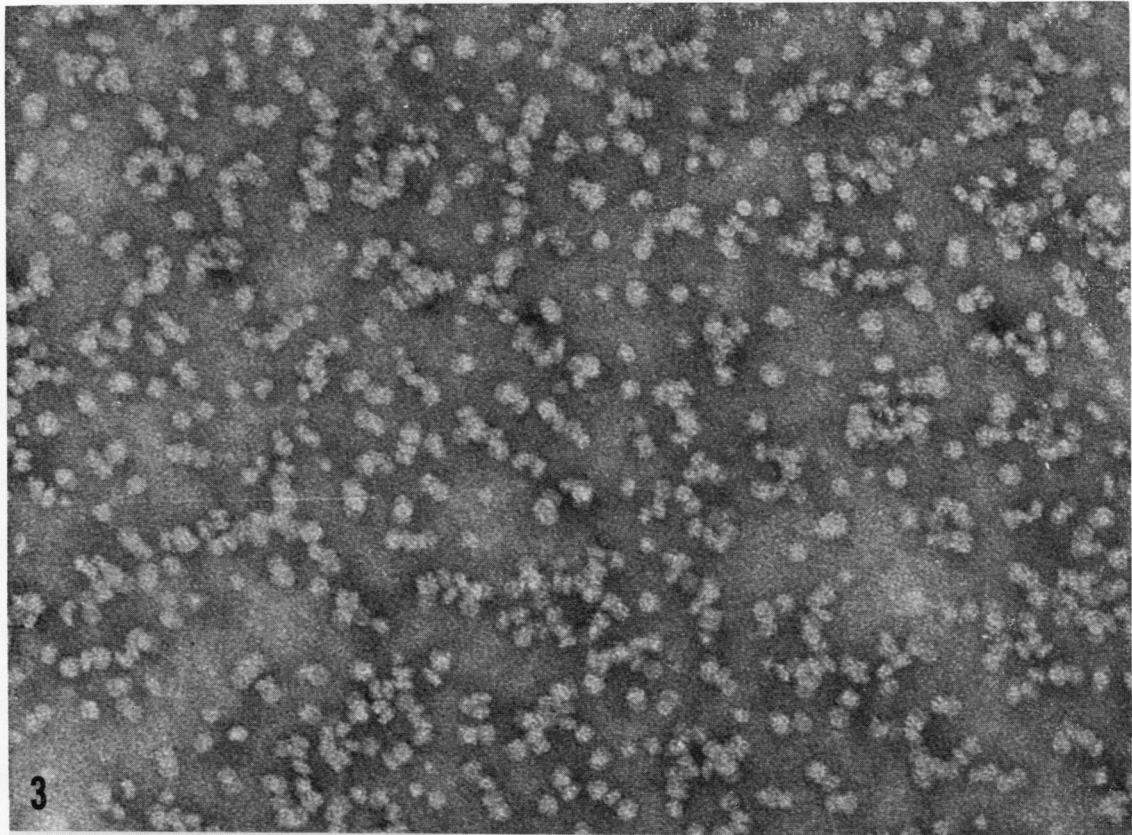


FIG. 3. Phycoerythrin fixed in glutaraldehyde and stained with phosphotungstic acid consists of a mixture of particles in various states of aggregation. Side views of the particles are clearly evident in most of the linear aggregates. 266,000 $\times$ .

FIG. 4. Phosphotungstic acid stained preparation extracted from the second phycoerythrin band obtained on polyacrylamide gel. Most are dimers. 266,000 $\times$ .

FIG. 5. Preparation from the third phycoerythrin band on polyacrylamide gel. Trimers are the predominant species. Stained with phosphotungstic acid. 266,000 $\times$ .

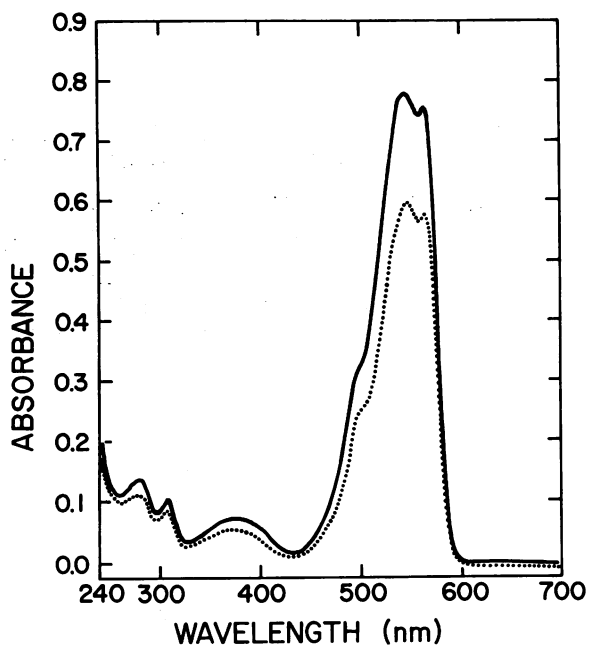


FIG. 6. Absorption spectra of phycoerythrin in 0.01 M phosphate buffer pH 6.8 (93  $\mu\text{g}/\text{ml}$  (—)), and a similar preparation (68  $\mu\text{g}/\text{ml}$ ) which had been treated in glutaraldehyde (.....). With glutaraldehyde treatment no shift in the absorption maxima is apparent.

from a through focus series, and because one can easily create artifacts with this technique.

Phycocyanin in its most stable form has a molecular weight around 200,000 (3, 12, 19, 20, 21), very close to that of phycoerythrin. Berns and Edwards (2) regard phycocyanin of this size to be the *in vivo* functional species. It is described as a ring with 6 distinct subunits, a central hole, and overall diameter of 130A (PTA staining). Phycoerythrin, a compact molecule with no definable subunits, and diameter of 101A, although designated as a monomer is believed to be functionally comparable. The ultrastructure of phycoerythrin is also quite distinct from phytochrome, which seems to exist as a tetramer or hexamer (5). Certain calculations can be made using some of our previous data. The phycobilisomes (10, 11) have a diameter of about 350A and are composed of closely associated particles. It has been calculated for *P. cruentum* that for every phycoerythrin molecule there are 40 chlorophyll molecules ( $1.4 \times 10^8$  chlorophylls to every phycobilisome) although the chlorophyll is not in the phycobilisomes. On a volume basis about 60 phycobiliprotein molecules could fit into a phycobilisome. This would be the case if the molecules were solidly packed. However, the electron micrographs show that they are not solidly packed because the negative stain can penetrate. It is reasonable to assume that each phycobilisome contains about 35 phycobiliproteins (phycoerythrin and phycocyanin) with phy-

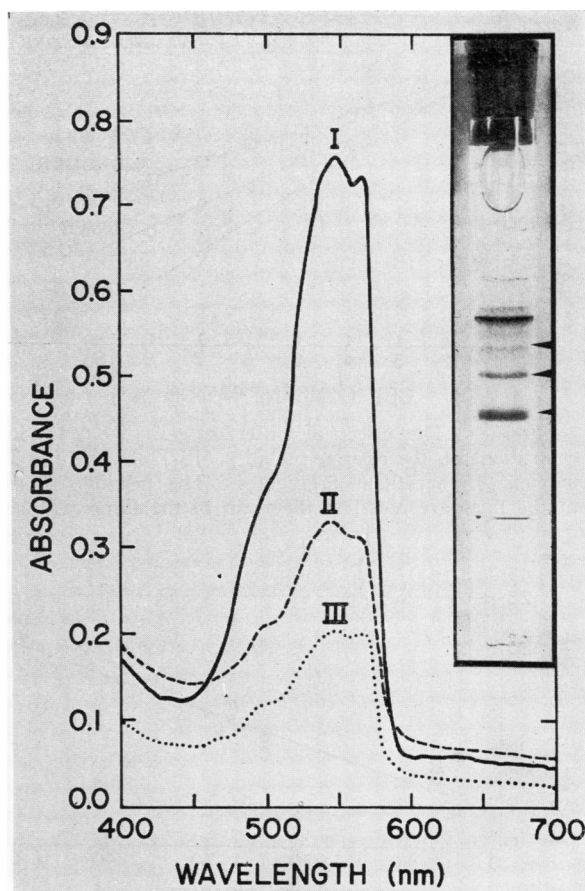


FIG. 7. Results of glutaraldehyde fixed phycoerythrin after disc electrophoresis on 5% polyacrylamide gel. The inset is a photograph of a stained gel with the monomer, dimer, and trimer bands marked on the right. The larger phycoerythrin aggregates were unable to penetrate into the small pore gel and have accumulated in the large pore gel, as well as at the interface between the 2 gel phases. Sample concentration was about 75  $\mu\text{g}$ . The wire marks the distance the dye front migrated. Absorption spectra in 0.001 M phosphate buffer pH 6.8 of the monomer (I), dimer (II), and trimer (III) bands after extraction from the gel have the same maxima (563, 545, and 500 nm shoulder).

coerythrin predominating. From these results it should now be possible to distinguish between several probable molecular organizations in phycobilisomes: a model in which phycocyanin is present as a core directly attached to the photosynthetic lamellae with a phycoerythrin shell, or a spoke model where chains of phycocyanin and phycoerythrin molecules are interspersed.

#### Acknowledgments

The author expresses appreciation to Dr. K. M. Towe, of the Paleobiology Department of the Smithsonian Institution, for the use of the electron microscope and associated facilities, and to Miss Claudia A. Lipschultz for her able technical assistance.

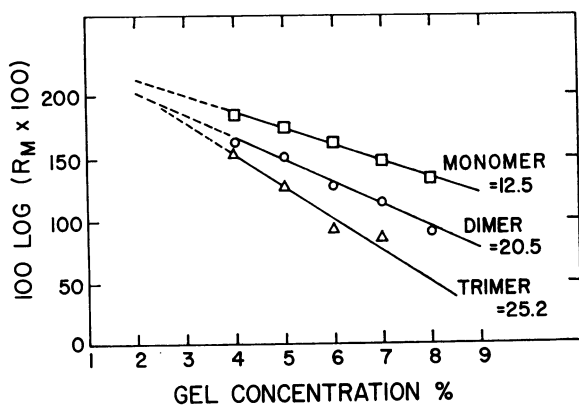


FIG. 8. The effect of different gel (4-8%) concentrations on the relative mobility ( $R_M$ ) of the 3 fastest moving phycoerythrin aggregates. The negative slopes of the lines are noted at the right of the figure.

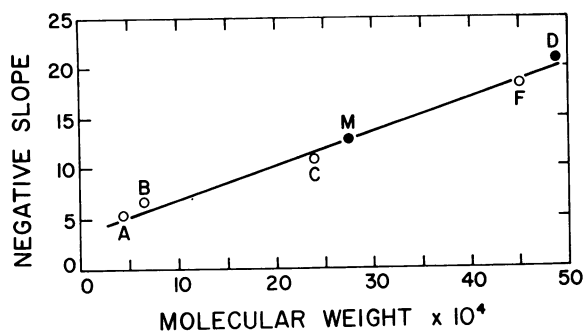


FIG. 9. The slope-molecular weight relationship of proteins of known molecular weight and the phycoerythrin monomer (M), and dimer (D). A-alpha amylase, B-bovine serum albumin monomer, C-catalase, F-ferritin.

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