PROPERTIES OF PHYCOBILINS FROM PORPHYRA NAIADUM*

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ABSTRACT

The phycobilin pigments were freshly extracted from *Porphyra naiadum* in the cold.

At least two types of phycoerythrin (I and II) can be distinguished by electrophoresis, chromatography, and spectral characteristics.

At pH 5.0 phycoerythrin II has a relatively large negative charge, while phycoerythrin I is nearly iso-electric. At pH 7.0, however, phycoerythrin I has the larger negative charge. Mobilities have been calculated by visual measurement of electrophoresis.

Phycoerythrin II can be converted to phycoerythrin I by storing at pH 7.0.

Chromatography indicates at least two types of phycocyanin as well.

The molecular weight of crystalline phycoerythrin has been determined several times using the pigment isolated from a number of red algae (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932; Eriksson-Quensel, 1938). In most of these investigations the extraction and crystallization procedures were lengthy and carried out under conditions conducive to change in the protein moiety of the phycoerythrin molecule. In some cases the problem is compounded by difficulty in extracting the pigment from the algae; (*e.g. Ceramium, Polysiphonia,* or *Antithamamnion*). In contrast to these, the phycobilins from *Porphyra naiadum* can be extracted rapidly (Airth and Blinks, 1956). The thallus is very delicate, and little bacterial contamination occurs during extraction. Yield of pigment is high (75 per cent).

Phycoerythrin, once extracted, has usually been crystallized by ammonium sulfate (Lemberg, 1928; Boresch, 1932), with yield reported to be quantitative. However, the phycobilins from *P. naiadum*, extracted for a short time (15 hours) in the cold with precautions taken to avoid basic extraction conditions, were not quantitatively crystallized by this method (Airth and Blinks, 1956). But if the pigment solutions were allowed to "age" at room temperature then phycoerythrin could be readily crystallized from them. This sug-

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gests a change in the protein portion of the phycoerythrin molecule. This possibility was further investigated, mainly by chromatographic and electrophoretic techniques.

Methods

The extraction and purification of phycobilins from *P. naiadum* have already been considered in detail elsewhere (Airth and Blinks, 1956). The algae were extracted with water at 5°C. for 15 hours. After extraction the algal residue was separated by centrifugation and the supernatant pigment solution dialyzed overnight at 1°C. in 0.1 M acetate buffer, pH 5.0. This dialyzed pigment solution constituted the "stock pigment solution." Neither during extraction nor dialysis did the pH of the pigment solution rise above 7.0.

Electrophoretic separations were carried out on the stock pigment solution in a Klett electrophoresis apparatus. Schlieren optics were not available in most of the work, and are difficult to use in the case of pigments: visual measurement of the fronts was carried out and presented few difficulties. The separation of the various phycobilins could be observed continuously. The movement of the pigment boundary was measured by sighting between front and rear scales to avoid parallax: movements of a millimeter could be detected. This method had two disadvantages, however: (*a*) colorless impurities were not detected, and (*b*) different pigment boundaries were not evident unless the color difference was large.

At the end of an electrophoretic run the cells were isolated and the per cent of the total pigment present in each was calculated from the following information: (*a*) the optical density of the original pigment solution, (*b*) the volume of the pigment introduced into the apparatus, (*c*) the volume of each cell (or if the cell was not completely filled by pigment, the volume occupied by it), (*d*) the absorption of the pigment solutions isolated from the various cells. By this method it was generally possible to account for at least 95 per cent of the total pigment initially introduced. (Partial precipitation sometimes occurred, especially in the center cell.)

The absorption spectra of purified phycoerythrin, phycocyanin, and allophycocyanin from *P. naiadum* have been published by French *et al.* (1956). B-phycoerythrin (Airth and Blinks, 1956) has a major absorption maximum at 545 mμ, C-phycocyanin at 615 mμ, and allophycocyanin at 655 mμ. These wave lengths have been used to analyze the per cent of total pigment present in each cell as described above, although in all cases the complete absorption spectra were determined from 400 to 700 mμ. It has been calculated that at 545 mμ in a fresh stock pigment solution, C-phycocyanin absorbed 9 per cent and allophycocyanin 3 per cent of the total light absorbed, while at 615 mμ B-phycoerythrin absorbed 2 per cent and allophycocyanin 12 per cent of the total light absorbed.

The following notation was adopted to identify the electrophoretic cell from which the pigment fractions were isolated: the +1 pigment fraction was obtained from the cell nearest the anode and the −1 fraction from the cell nearest the cathode. The +2 pigment fraction was isolated from the cell which was second nearest the anode and the −2 fraction from the cell second nearest the cathode (see Fig. 1). The C fraction was isolated from the center cell, between the −2 and +2 electro-

phoretic cells. Unless otherwise stated the −1, −2, and C cells of the apparatus were filled with pigment at the beginning of the experiment.

The chromatographic separations were fashioned after the researches of Swingle and Tiselius (1951).

RESULTS

Freshly prepared stock pigment solution was separated electrophoretically at pH 5.0; all pigment movement was toward the anode. The absorption spectra of the various fractions are presented in Fig. 1. Judging from these, the purple +1 fraction contained phycoerythrin, phycocyanin, and allophycocyanin;

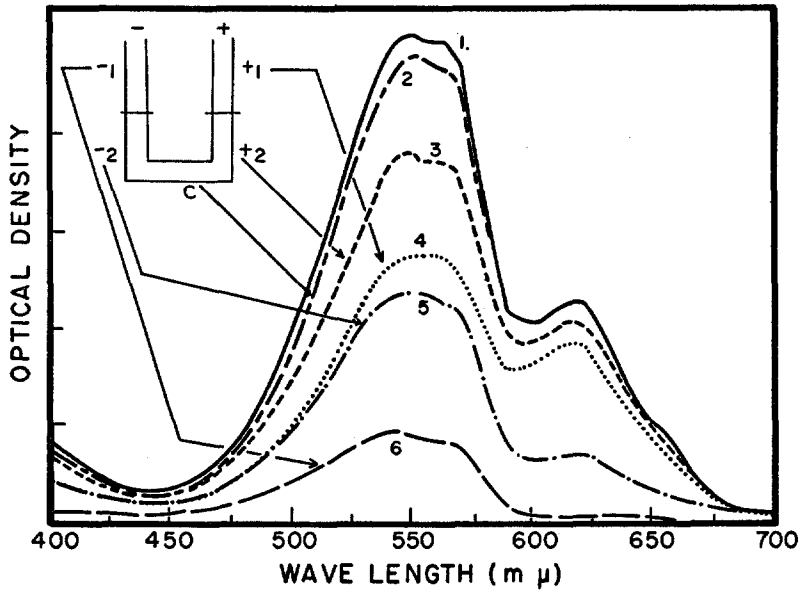


FIG. 1. Absorption spectra of the fractions isolated by electrophoresis at pH 5.0. (1) original dialyzed solution; (2) C fraction; (3) +2 fraction; (4) +1 fraction; (5) −2 fraction; (6) −1 fraction.

the +2, C, and −2 fractions contained the same pigments; while the red −1 fraction contained only phycoerythrin. The homogeneity of the latter fraction was demonstrated more convincingly with concentrated solutions. The absorption spectrum of the dialyzed pigment solution before electrophoresis is also presented. From such data it was concluded that there were at least two types of phycoerythrin in the stock pigment solution; one that has a relatively large negative charge at pH 5.0 (henceforth designated phycoerythrin II) and one that has a small negative charge (phycoerythrin I).

The ratios of the optical density at 545 mμ to that of 615 mμ for the various fractions are as follows:

	Ratio
Dialyzed pigment solution	2.19
+1	1.45
+2	1.86
C	2.10
−2	3.72
−1	15.6

From these values it can be concluded that phycocyanin has a relatively high anodal movement at pH 5.0, and that the proportion of phycoerythrin increases toward the −1 cell (in which it is the major component). It is also worthy of note that of the total phycoerythrin present, only 5 to 10 per cent was in the form of the nearly immobile phycoerythrin I. This observation was consistent with attempts to crystallize phycoerythrin and indicated that phycoerythrin I is crystallizable by isoelectric precipitation at pH 4.5 while phycoerythrin II is not.

In a separate experiment an electrophoretic separation was carried out on the stock pigment solution from which the phycoerythrin I had been removed by crystallization with acid. The supernatant at pH 5.0 consisted almost entirely of phycoerythrin II and phycocyanin; no phycoerythrin I was electrophoretically detected.

When however, freshly prepared stock pigment solution was dialyzed overnight at 1°C. in phosphate buffer (of ionic strength 0.1) at pH 6.98 a different electrophoretic behavior resulted. The separation obtained is diagrammed in Fig. 2. The colored fractions were separated as carefully as possible (with a hypodermic needle and syringe) and studied spectrophotometrically. Their absorption spectra are presented in Fig. 2. The lavender and orange-red fractions are very similar spectrally, with possibly a slight shift in the absorption maxima and a greater degree of "shoulder" in the 560 mμ region of the orange-red fraction. Since the absorption spectrum of the orange-red fraction is similar to that of phycoerythrin I, it is concluded that phycoerythrin I at pH 5.0 is less negatively charged than at pH 7.0. Conversely if phycoerythrin II is represented in the deep purple fraction, its pH-charge characteristics are the opposite, in that at pH 5.0 it was more negatively charged than at pH 7.0.

The bright blue fraction isolated by electrophoresis at pH 7.0 is probably phycocyanin with a considerable phycoerythrin impurity. This fraction is somewhat reminiscent of the floridean R-phycocyanin (Kylin, 1912; Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Lemberg, 1930; Haxo *et al.*, 1955), but the latter has its first absorption maximum around 550 mμ, instead of at 570 mμ as in this fraction. Purified phycocyanin from *P. naiadum* isolated by chromatography (see below) has a single absorption maximum at approximately 615 mμ. It is possible, however, by us-

ing the technique of Vandenbelt and Henrich (1953), to draw a summation curve of a mixture of phycoerythrin and phycocyanin which has absorption characteristics very similar to those reported for R-phycocyanin. The possibility that all phycocyanins have only one absorption maximum around 615 mμ has been suggested by Boresch (1932) and would seem worthy of further consideration.

The possibility that the relative mobilities of phycoerythrin I and phycoerythrin II are mutually reversed at pH 5.0 and 7.0 can also be seen from Fig.

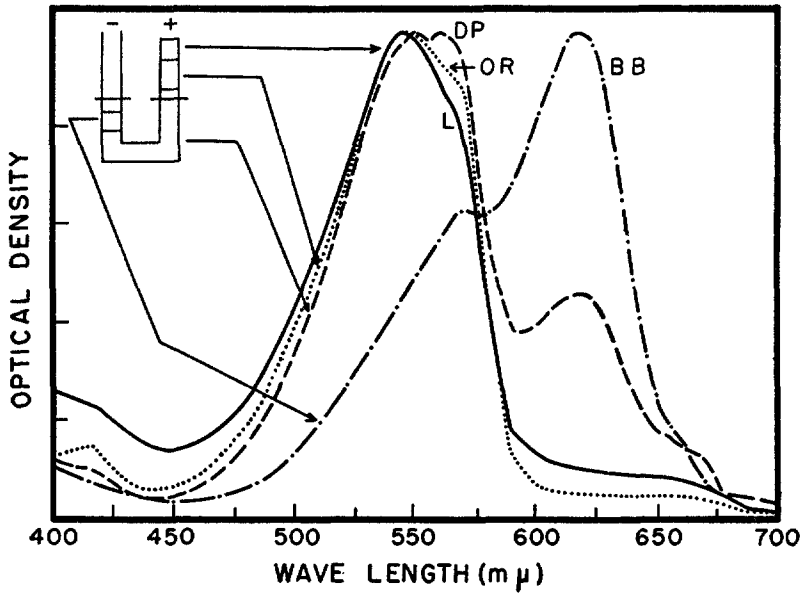


FIG. 2. Absorption spectra of fractions isolated by electrophoresis at pH 7.0. *L*—lavender; *OR*—orange-red; *DP*—deep purple; *BB*—bright blue.

3. In these experiments the −2, C, and +2 cells were filled at the beginning of the Tiselius run. These curves represent the absorption maxima (540 to 570 mμ) between pH 4.98 and 7.25 of the +1 and −2 fractions after electrophoretic separation of the stock pigment solution. It will be noticed that at pH 4.98 the pigment in the +1 cell has its major peak at 567 mμ and a smaller "shoulder" at 555 mμ. This fraction represents phycoerythrin II. The −2 fraction, at the same pH, has its major peak at 546 mμ and the center of a "shoulder" at 563 mμ. At pH 5.47 the situation is altered, for in the +1 cell the major peak is at 550 mμ and a minor peak at 562 mμ; while the −2 cell pigment does not have an evident major peak, but two maxima. When electrophoretic separation is made at pH 7.25 the major peak at 549 mμ and a "shoulder" at 561 mμ are

represented by the pigment isolated from the +1 cell while the pigment from the −2 cell has a major maximum at 562 mμ with the center of a "shoulder" at 554 mμ. Through the pH region of 4.98 to 7.25 the positions of the major

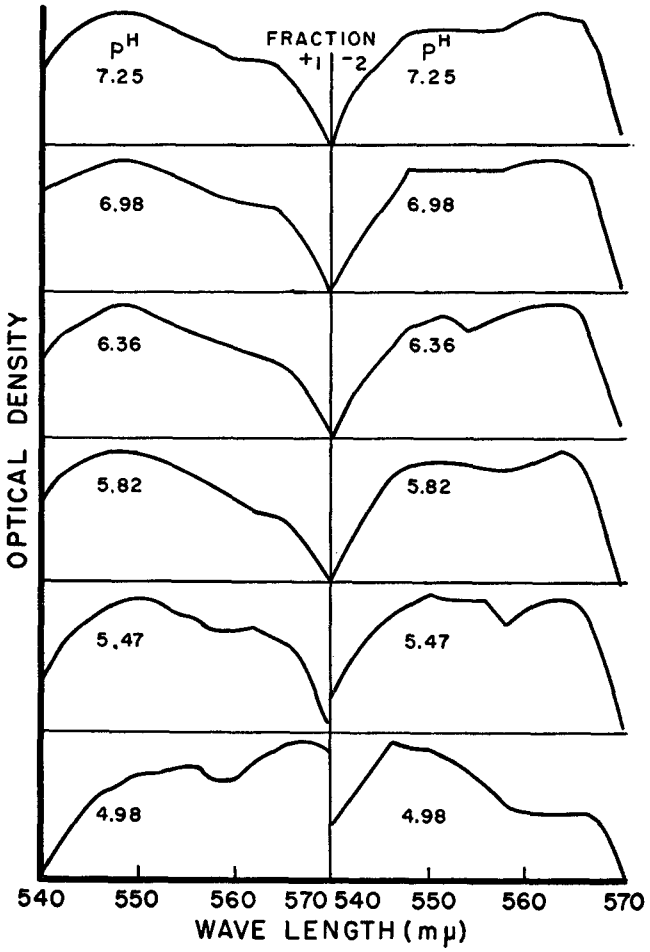


FIG. 3. Absorption maxima (from 540 to 570 mμ) of +1 and −2 electrophoretic fractions between pH 4.98 and 7.25. ΔO.D. in each curve is about 0.05.

peaks have been reversed. Thus at pH 4.98 the +1 pigment has a major maximum at 567 mμ whereas at pH 7.25 the 567 mμ maximum is shown by the pigment isolated from the −2 cell. Correspondingly, at pH 4.98 the pigment from the −2 cell has its major absorption at 546 mμ whereas similar absorption characteristics are exhibited by the pigment isolated from the +1 cell at pH 7.25.

It has already been reported that if the stock pigment solution is stored for several days at pH 7.0, a greater yield of crystalline B-phycoerythrin could be obtained (Airth and Blinks, 1956). Apparently phycoerythrin II is slowly converted to phycoerythrin I at neutrality. This conversion was therefore studied in more detail. Fresh stock pigment solution was divided into two parts; one was dialyzed overnight in 0.1 M phosphate buffer pH 7.0, the other in 0.1 M acetate buffer at pH 5.0 (both at 1°C). The two fractions were then stored in the dark at 5°C. and on various days aliquots were taken for electrophoretic study. Both aliquots were dialyzed overnight in 0.1 M acetate buffer pH 5.0

TABLE I

Change in Electrophoretic Distribution of Stock Pigment Solution kept at pH 5.0 and pH 7.0

Per cent of total pigment from cells indicated.

Time	pH 5.0		pH 7.0	
	Phycoerythrin II from +1 cell	Phycoerythrin I from −1 cell	Phycoerythrin II from +1 cell	Phycoerythrin I from −1 cell
days				
1	23.4	7.6	24.0	2.8
2	22.0	7.3	24.3	2.8
3	25.1	6.2	21.0	5.1
4	28.2	6.1	8.5	15.7
5	29.8	5.9	11.9	13.0
7	26.9	3.3	—	—
8	—	—	14.9	8.7
15	—	—	6.5	19.7
16	26.6	4.4	—	—
22	—	—	5.9	24.7
24	22.2	5.7	—	—
29	—	—	4.2	25.0
31	22.8	5.5	—	—

(the pH 7.0 aliquots being thereby restored to pH 5.0) at 1°C. for buffer equilibrium and separated in the Tiselius apparatus the following day. After electrophoresis each fraction was isolated and studied spectrophotometrically. The amount of phycoerythrin I in the −1 cell was compared to the phycoerythrin II in the +1 cell as explained above. The presence of phycocyanin in the +1 cell fraction has been corrected for. The results of this experiment are presented in Table I.

The data indicate that the highly mobile phycoerythrin II isolated from the +1 cell, in the pH 5.0 fraction constituted about 25 per cent of the total pigment over the entire 31 days; whereas in the pH 7.0 aliquots, the amount of phycoerythrin II was reduced from about 25 per cent of the total pigment to about 5 per cent in 29 days. Conversely, while the phycoerythrin I in the

pH 5.0 fraction was about 6 per cent of the total pigment over the experimental period, in the case of the pH 7.0 aliquots the amount of phycoerythin I increased from 3 per cent to 25 per cent. Evidently phycoerythrin II is fairly stable at pH 5.0 while at pH 7.0 it is converted into phycoerythrin I.

The conversion of phycoerythrin II to phycoerythrin I was also studied in a somewhat purified fraction. The contents of the +1 cell were removed after electrophoretic separations at pH 5.0, several runs being necessary to get a sufficient volume. The pooled samples were divided into two equal parts, one of which was dialyzed for about 12 hours against 0.1 M acetate buffer, and the other against 0.1 M phosphate buffer at pH 7.0, both at 1°C. After this time, both fractions were dialyzed for an additional 12 hours in 0.1 M acetate buffer, pH 5.0, and at the same temperature. Electrophoresis was then carried out on both solutions. The per cent of phycoerythrin present in each cell is given in Table II.

TABLE II

Change in Electrophoretic Distribution of Phycoerythrin II Kept at pH 7.0 and 5.0 for Approximately 12 Hours

Per cent of total pigment in	pH 5.0	pH 7.0
+1	28.2	22.3
+2	33.1	32.1
C	26.3	25.1
−2	10.4	9.4
−1	2.0	11.1

The +2, C, and −2 cells show very little difference between the two fractions. The +1 cell contained considerably less pigment from the pH 7.0 fraction than from the pH 5.0 fraction; in the −1 cell the opposite was found. Thus greater conversion of highly mobile phycoerythrin II into less mobile phycoerythrin I was found at pH 7.0, and the rate of conversion is greater when phycoerythrin I is initially absent. Table I indicates that it took from 4 to 8 days for a stock solution at pH 7.0 to produce approximately 11 per cent of the total pigment as phycoerythrin I. However, after electrophoretic removal of the phycoerythrin I only 12 to 24 hours were needed for the production of this amount of new phycoerythrin I. Evidently some kind of equilibrium is involved.

In order to obtain a more complete description of the electrical properties of the phycobilin pigments, a pH-mobility experiment was carried out. The visual method of measuring mobility leaves something to be desired, but the data are in general agreement with some results using schlieren optics. The buffers were those recommended by Conway (1952), with the final solution

being adjusted to an ionic strength of 0.1. In these experiments the C, −2, and +2 Tiselius cells were filled with dialyzed pigment at the beginning of the experiment. Electrophoresis was carried out until the +1 cell was completely filled with pigment. The pH mobility curves of the various fractions are presented in Fig. 4.

One of the difficulties of conducting such experiments with mixed pigment solutions is detecting, isolating, and identifying the various pigment boundaries and fractions. At pH 5.0 two fractions were usually present which were sufficiently different in color to follow readily. At pH 7.0, as already mentioned, four different pigment boundaries were detectable by eye. The various

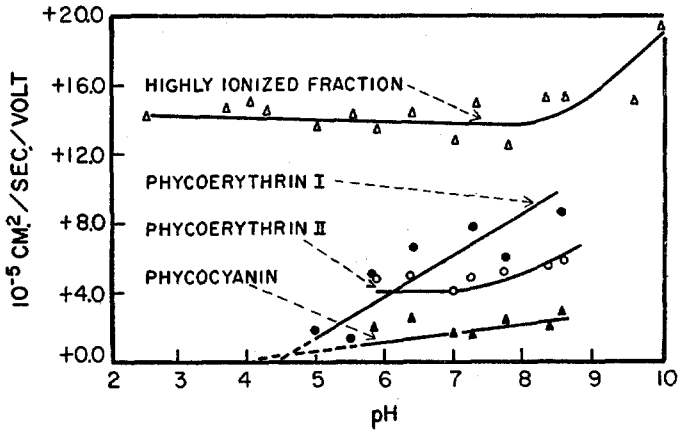


FIG. 4. pH mobility curves of fractions in stock pigment solution. Highly ionized fraction—open triangles; phycoerythrin I, closed circles; phycoerythrin II, open circles; phycocyanin, closed triangles.

fractions were isolated and identified spectrophotometrically. The lavender and orange-red pigment fractions became visible as ascending boundaries first at about pH 6.0. The latter fraction was no longer visible at pH 9.52 and is either bleached or its mobility has increased to such an extent that it is no longer visibly distinguishable from the lavender fraction. The least mobile blue fraction (phycocyanin) first became evident at pH 6.36 and was found in all subsequent separations until the electrophoresis at pH 9.97 when it was absent. The lavender and blue fractions were probably present at pH 5.0 but indistinguishable from the deep purple fraction.

From the theory of electrophoresis it is apparent that only the fastest (lavender) and slowest (bright blue) fractions can possibly be homogeneous (*cf.* Fig. 2). The next fastest (orange-red) fraction will definitely contain the lavender compound as well as phycoerythrin I. The deep purple fraction will be the most

complex, containing the lavender compound, phycoerythrin II, phycoerythrin I, phycocyanin, and allophycocyanin. The predicted complexity of this fraction is attested to by its absorption spectrum. The chief difficulty was to determine which pigment fraction the various electrophoretic boundaries represented. As the absorption spectra of the isolated fractions represent complex mixtures in some cases the correlation of electrophoretic boundary and pigment fraction has necessitated subjective interpretation. The fact that the relative mobilities of phycoerythrin I and II differ at pH 5.0 and 7.0 also increased the uncertainty of interpreting the data. Fig. 4 has been drawn with these difficulties in mind. It will be seen that the curves for phycoerythrin I and II cross at about pH 6.4.

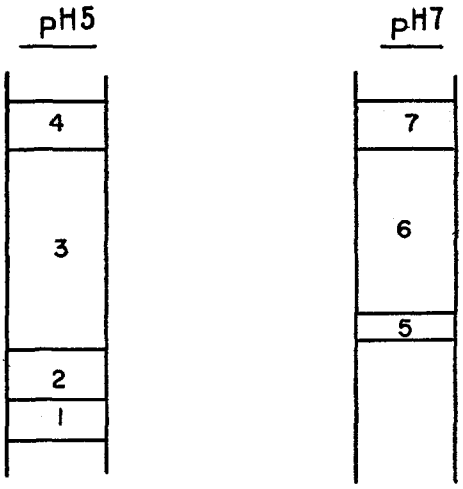
The fact that there is a pigment fraction which has a high anodal mobility at a low pH value is not characteristic of a typical protein and it has been concluded that there is a "highly ionized fraction" involved. This fraction has a relatively large negative charge throughout the entire pH range studied. It is the lavender fraction of Fig. 2. If the data are interpreted in this manner, then phycoerythrin I and phycocyanin have the typical pH mobility characteristics of proteins, as shown in Fig. 4. Phycoerythrin II is unusual in that it has no iso-electric point within the pH range studied (pH 4 to 9); in this it resembles the highly mobile lavender fraction. No explanation is at hand for this, except to postulate some highly ionized radical such as phosphate or sulfate on the molecule. Attempts to demonstrate these strongly dissociated radicals have however, so far proven fruitless (Jones and Blinks, 1957).

Possibly offering an explanation for the mobile pigment fraction is the extremely fast anodal transport of a greenish or yellowish component observed in extracts from macerated red algal tissue which have not been filtered or centrifuged. This turbid, particulate fraction moves rapidly ahead of the dissolved pigments; it may represent chloroplast fragments from which the phycobilins have been separated (chlorophyll and carotenoids seemed to be present). There may be some of this substance (combined with phycobilins) present in solution in filtered, centrifuged extracts of *Porphyra naiadum.*

Chromatography

Chromatographic separation of the pigments present in a freshly prepared stock pigment solution was also carried out. The adsorption pattern depended on the pH at which the column was eluted. Fig. 5 is a diagram of the fractions which separated on columns when the stock pigment solution was eluted at pH 5.0 and 7.0. Also presented are the concentrations and pH of the eluant and the absorption maxima of each band. In both cases there was a type of phycoerythrin that was readily eluted and a type that was not. The lavender fraction (No. 3) of the pH 5.0 column was found to be electrophoretically homogeneous and had the high mobility characteristics of phycoerythrin II. When this frac-

tion was rechromatographed it separated into phycoerythrin and phycocyanin. It is also worthy of note that the phycoerythrin of the lavender fraction on the



No. 1. BLUE, PC, λ_{max}. 615–617.5 mμ.
M ACETATE, pH 5.0

No. 2. PINK, PE, λ_{max}. 545mμ, 560–565mμ "SHOULDER"
M ACETATE, pH 5.0

No. 3. LAVENDER, PE + PC, λ_{max}. 565mμ 615mμ.
2M ACETATE, pH 5.0

No. 4. DARK BLUE, APC, λ_{max} 655mμ.
M/10 PHOSPHATE. pH 7.0

No. 5. PINK, PE, λ_{max} 545mμ, 560–565mμ, "SHOULDER"
M/20 PHOSPHATE pH 7.0

No. 6. LAVENDER, PE + PC + APC, λ_{max} 545mμ; 565mμ "SHOULDER"
615mμ, 655mμ, M/20 PHOSPHATE pH 7.0

No. 7. DEEP BLUE, APC, λ_{max} 655mμ, M/10 PHOSPHATE pH 7.0

FIG. 5. Chromatographic pigment separation at pH 5.0 and 7.0. Abbreviations: *PC*, phycocyanin; *PE*, phycoerythrin; *APC*, allophycocyanin.

pH 5.0 column required 2 M acetate buffer to elute it while the same pigment in the pink region required only molar buffer to move it. This is another indication that the two phycoerythrins are different, presumably in charge and resulting affinity for the adsorbent.

Tiselius (1955) and Tiselius, Hjertén, and Levin (1956) reporting on the chromatographic separation of phycoerythrin from *Ceramium* have come to the conclusion that this alga also has at least two and possibly three different types of phycoerythrin. Similar results with *Aphanizomenon* and *Microcystis* indicate that there are two and possibly three kinds of C-phycocyanin present in the blue-green algae. These experiments demonstrate very clearly that the separation of the various constituents depends upon the concentration of the eluant.

At pH 5.0 chromatographic separation of *P. naiadum* stock solution yields a phycocyanin fraction which is eluted off with 1 M buffer and another which is eluted at 2 M. This suggests that there may be more than one type of phycocyanin present. Electrophoretic separation gave additional evidence of this. Occasionally when the stock pigment solution was separated electrophoretically at pH 5.0 there was a blue fraction which had a mobility even less than that of phycoerythrin I. Sufficient quantities of this fraction were never obtained for spectrophotometric study. It seems to be readily bleached and hence not to be found in every separation. Should this fraction prove to be phycocyanin, the charge characteristics of this pigment would parallel those of phycoerythrin: one form with a large negative charge and one with a relatively small negative charge, at pH 5.0.

DISCUSSION

The experiments indicate that a fresh extract of *Porphyra naiadum* has at least two types of phycoerythrin and possibly a minimum of two types of phycocyanin. These pigments were all obtained with special care to control time, temperature, and pH during extraction. It is difficult to decide which pigment or pigments are "natural," since the definition of a native protein is vague. Although two types of phycoerythrin are found we do not really know whether this is due to the method of extraction and subsequent treatment or represents the situation in the algae. If several different pigment types also exist in the plant, then the question of their biological significance arises.

The photosynthetic function of the phycobilin pigments is now well demonstrated (Haxo and Blinks, 1950; Duysens, 1951; French and Young, 1952; Yocum and Blinks, 1954) and the types of electronic energy transfer mechanism have been reviewed by Rabinowitch (1951). The question of whether both types of phycoerythrin in *P. naiadum* are photosynthetically active is not clear at this time. Since in a freshly extracted pigment solution at least 90 per cent of the total phycoerythrin present is phycoerythrin II it seems most certain that this pigment must be active in photosynthesis, since efficiency is good in its region of absorption. Whether phycoerythrin I is also active is not so clear—if indeed it exists in the cell as such.

The transfer of light energy from phycoerythrin to phycocyanin requires that these two molecules be in close proximity (Rabinowitch, 1951). When the

highly ionized phycoerythrin II fraction was first isolated, and it was found always to contain phycocyanin, it was suggested that phycocyanin and phycoerythrin might be "complexed" in some fashion,—possibly with the phycoerythrin and phycocyanin chromophores attached to the same protein. While this hypothesis has not been disproved, a more rational interpretation of these and other experiments is that the fraction consists of two separate pigments, phycoerythrin II and "phycocyanin II" each with high mobility. Since there are two types of phycoerythrin in the pigment solution, (and further investigation may reveal others) the same may well be true of phycocyanin. Electrophoresis through greater distances, chromatography with greater column capacity or eluants of different concentration ranges, may eventually solve this problem.

We have already described (Airth and Blinks, 1956) a new pigment from *P. naiadum*, designated B-phycoerythrin; it has only one main absorption maximum at 545 mμ. This pigment has the same absorption maximum over a broad pH range and after several recrystallizations is more resistant to bleaching by hydrogen peroxide than is the phycoerythrin of the stock pigment solution. Phycoerythrin I and B-phycoerythrin are identical judging from the absorption spectra and the fact that both can be isolated by isoelectric crystallization. B-phycoerythrin has charge characteristics similar to those of phycoerythrin I and is homogeneous in electrophoresis and ultracentrifugation. The main differences between phycoerythrin I and II are those of charge and the fact that phycoerythrin II seems to have its major absorption maximum around 565 mμ. (The latter may be due to the influence of the phycocyanin in the solution however).

In the past the classification of phycobilin pigments has been based mainly on their spectral characteristics. While this is simple, additional characteristics may have to be used in the future. Indeed a completely new system of classifying these pigments might eventually be necessary.

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