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Phycocyanobilin. Structure and Exchange Studies by Nuclear Magnetic Resonance and Its Mode of Attachment in Phycocyanin. A Model for Phytochrome*

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ABSTRACT: Phycocyanobilin, the prosthetic group of phycocyanin, has been isolated in high yield by methanol extraction. Its structure has been determined by nuclear magnetic resonance and mass spectral studies to be mesobiliverdinoid, but with an ethylidene group at position 2. The methyl hydrogen atoms of this ethylidene group, as well as of methine bridge *c* and the hydrogen at position 1, α to a carbonyl group, have been found readily exchangeable. Present evidence indicates at least two possible ester modes of attachment of bilin to apoprotein, and one of these offers a model for the photochromic behavior of phytochrome.

Phycocyanin, a photosynthetic protein present in blue-green algae, is one of an important group of plant proteins that contain a bile pigment prosthetic group. The structure of the prosthetic group of phycocyanin, phycocyanobilin (PCB),[1] was first described by Lemberg (Lemberg and Bader, 1933; Lemberg and Legge, 1949) as a linear tetrapyrrole compound. A more definitive characterization of phycocyanobilin has been subsequently severely hampered by the lack of a good preparative procedure and the apparent lability of the compound (*cf.*, for example, Ó hEocha, 1963, 1966). It was found recently, however (Fujita and Hattori, 1962, 1963), that refluxing whole algae with methanol would liberate phycobilins. Ó Carra and Ó hEocha (1966) and Siegelman *et al.* (1966) have used this observation as the basis of their methods for the liberation of phycobilins from purified protein. The preparative procedure reported here is a considerable modification of those previously reported and allows very high yields of phycocyanobilin.

In our work, we have isolated phycocyanobilin from C-phycocyanin in 40–50% yield. Our product is essentially chromatographically pure and shows only traces of other pigments. In view of the possible lability of this bilin, we felt it of overriding importance to avoid any extensive chemical or chromatographic processing. To this end, we have confined ourselves to mild procedures involving solvent extraction and have worked with the free acid so obtained rather than resorting to esterification. From data obtained with material isolated by our procedure, we propose a structure for phycocyanobilin (Figure 7A). This structure is very similar to, but not the same as, that (Figure 7C) reported by other workers (Cole *et al.*, 1967; Rüdiger *et al.*, 1967) and provides a basis for the chemistry of the ethylidene functional group. Also, the placement of the ethylidene group at position 2 in the PCB molecule is established with some rigor. Further, with respect to the mode of attachment of phycocyanobilin to the apoprotein, we present data that suggests two possibilities: the first, an ester linkage to serine; the second, an attachment involving a C–C bond directly to the peptide backbone that is labilized by rupture of an ester bond. The second possibility appears to offer an interesting model for the photoresponses of phytochrome. A preliminary report of some aspects of this work has already appeared (Crespi *et al.*, 1967).

Experimental Section

Isolation of Phycocyanin. C-phycocyanin was isolated from *Phormidium luridum* or *Synechococcus lividus* grown in H_2O or 99.8% D_2O (DaBoll *et al.*, 1962). The protein was purified by ammonium sulfate fractionation to a purity index (ratio A_{620}/A_{280}) of 3.3–4.0 (Hattori *et al.*, 1965a). For experiments involving proteolytic digestion, further purification was effected by

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PCB, phycocyanobilin; HMS, hexamethyldisiloxane; TFA, trifluoroacetic acid; DPCB, deuterated PCB.

passage through an Ecteola column (Hattori *et al.*, 1965a). The protein used in the enzyme experiments had purity indices of 4.8–5.0.

Isolation of Phycocyanobilin. A typical procedure is outlined below. The quantity of protein processed may be scaled up or down in a roughly linear fashion. A 2–6% solution of phycocyanin (2–4 g of protein) is dialyzed (at 5°) against 0.01 M phosphate buffer (pH 6.9). The buffered solution is diluted with methanol to a concentration of 90% methanol and refluxed for 18 hr under a nitrogen blanket. The blue solution is then centrifuged to remove the bulk of the pale blue-gray protein residue, evaporated to 200–300 ml on a rotary evaporator (35–40°), and centrifuged (15,000*g*, 10 min). The clear, blue solution is then extracted two or three times with petroleum ether (bp 20–40°) and the yellow-brown petroleum ether extracts are discarded. Then, *ca.* 200 ml of freshly destabilized chloroform[2] is added, and the mixture is acidified with 2–3 ml of glacial acetic acid and washed three or four times with copious amounts of sodium chloride solutions. (Water containing about 10% salt is used to prevent formation of emulsions. A little methanol may be added to the salt water to prevent complete removal of methanol from the chloroform layer, as phycocyanobilin has only limited solubility in chloroform washed thoroughly with water.) The blue chloroform solution is evaporated to dryness at 30° and the residue is dissolved in 8–10 ml of chloroform containing 2.5% methanol, filtered through a fine glass frit, and then dried by rotary evaporation at 25°. This residue is then taken up in about 4 ml of chloroform–methanol, precipitated by addition of seven volumes of petroleum ether, and centrifuged, and the centrifugate is washed with petroleum ether. The solid is dried with a slow stream of nitrogen, further dried by pumping on a vacuum line, and then stored at 5° in the dark in evacuated glass vessels. Yields are 40–50% of the calculated on the assumption that the bilin constitutes 4% (w/w) of phycocyanin. *Anal.* Calcd for $C_{33}H_{38}N_4O_6$: C, 67.7; H, 6.54, N, 9.55.[3] Found by combustion analysis (Crobaugh Laboratories): C, 65.27, 65.38; H, 7.10, 7.30; N, 9. 23, 9.12. Amino acids analysis showed less than 0.5% contamination with amino acids, with no amino acid in particular abundance. No melting is observed to 300°.

Instrumentation. Proton magnetic resonance spectra were determined with a Varian HA-100 spectrometer equipped for repetitive scanning. Unless otherwise specified, all chemical shifts are given in parts per million (δ) from internal hexamethyldisiloxane (HMS, 8%). Pyridine-d_5 was our usual solvent. With trifluoroacetic acid as solvent, the carboxyl protons were used as an internal lock, and chemical shifts were calculated from external HMS. Absorption spectra in the visible were taken with a Cary 14 spectrophotometer. Amino acid analyses were performed with a Beckman–Spinco Model 120 amino acid analyzer.

[2] The presence of ethanol in the chloroform used in processing results in the formation of ethyl esters of PCB.

[3] If one assumes a PCB monohydrate, the calculated values are: C, 65.7; H, 6.68; N, 9.29.

TABLE 1: Absorption Maxima (millimicrons) of Phycocyanobilin in Chloroform.

Ethanol Content of Chloroform (%)	γ_1	A_1[a]	λ_2	A_2/A_1
0.0	645	0.58	368	1.93
0.45	610	0.48	366	2.52
0.75	603	0.51	366	2.50
0.75[b]	602	0.52	364	2.28
3.7	595	0.47	366	2.62

[a] Positions of absorption maxima of PCB, free acid, are highly concentration dependent, so we have here compared solutions of roughly equal absorbance. If the absorptivity changes much with solvent some error will be introduced, but the trend above seems clear. [b] Phycobilin-630 prepared according to the method of Ó hEocha (1963).

Chromatography. Three solvent systems were used to analyze our bilin preparations. Solvent system A is a mixture of butanol–pyridine–water (3:1:1). Solvent system B is a mixture of butanol–pyridine–tetrahydrofuran (4.5:1:6), to which 2% water is added. Solvent system C is a mixture of toluene–acetic acid–water (4:1:1). System C was found very difficult to use in humid weather. Chromatography was carried out at room temperature (nominally 23°) on glass plates coated with Adsorbosil 3 or 5 (Anspec). Depending on conditions of temperature and humidity, R_F values varied by ±0.03 unit.

Proteolytic Digestion. Phycocyanin digestions were carried out at 35–37° with trypsin (Calbiochem), α-chymotrypsin (Worthington), and carboxypeptidases A and B (Worthington). Trypsin and chymotrypsin were added periodically over a period of 1 hr to the extent of 3% each of the amount of phycocyanin being digested. Carboxypeptidases A (3%) and B (1%) were then added, and digestion was allowed to continue for 15–20 min. The pH was maintained at 8.4 ± 0.4 with 0.01 M tricine buffer (Good *et al.*, 1966). The digest was then freeze dried.

Gel Filtration. The Sephadex G-15 (Pharmacia) column was 2.5 × 22 cm. All runs were made with 0.02 M tricine buffer (pH 8.2) at 5°. A typical load corresponded to 10 mg of phycocyanin. The flow rate was 15–18 ml/hr and 3-ml fractions were collected.

Preparation of Violinoid Derivative of Phycocyanobilin. Phycocyanobilin was dissolved in a mixture of 50% methanol and 50% 0.1 M bicarbonate buffer (pH 10.0) to give a 2% solution. This solution was heated to 40–50° for 1–2 hr. The reaction mixture was streaked onto plates coated with 1.25 mm of Adsorbosil-5, and the plates were developed with solvent system A. The main product, purple material of R_F 0.43 and a strong

TABLE II: Absorption Maxima (millimicrons) of Phycocyanobilin and Phycocyanin in Aqueous Systems.

Pigment System	At pH 5.4 ± 0.1[a]			At pH 8.1 ± 0.1[b]		
	λ_1	λ_2	A_2/A_1	λ_1	λ_2	A_2/A_1
Phycocyanobilin	635	361	2.00	600	361	2.90
Digested phycocyanin	626[c]	349[c]	1.63[c]	592	350	2.65
Phycocyanin in 7 M urea	655	355	1.09	598[b]	355	2.19
Phycocyanobilin in 7 M urea	680	372	1.48	622	368	3.04

[a] 0.01 M acetate buffer. [b] 0.02 M tricine buffer except urea solutions, 0.01 M tricine. [c] In 0.1 M acetate.

orange-pink fluorescence, was removed from the plate with a zone collector and eluted with methanol. (We have also used the inverted dry-column technique of Bhalla *et al.* (1967) for this purpose with good results.) The methanol solution was filtered through a fine frit, evaporated to a small volume (8–9 ml), and centrifuged for 30 min at 80,000*g*. The purple solution was then taken to dryness, dissolved in chloroform–2.5% methanol, acidified with 1 drop of acetic acid, and washed several times with salt (NaCl) water. Isolation of the product was then continued as for phycocyanobilin.

Reduction Experiments. Phycocyanobilin was dissolved in glacial acetic acid in the presence of solid sodium hydrosulfite. The mixture was heated with stirring to 55–60° for 1 min to give a clear yellow solution. The yellow solution was decanted, diluted with chloroform, and washed with salt (NaCl) water. The yellow chloroform layer was evaporated to dryness and redissolved in methanol or chloroform for chromatographic analysis. Native or digested phycocyanin was reduced by treating aqueous phosphate buffer solutions or 0.1 M Tris buffer solutions with solid potassium borohydride.

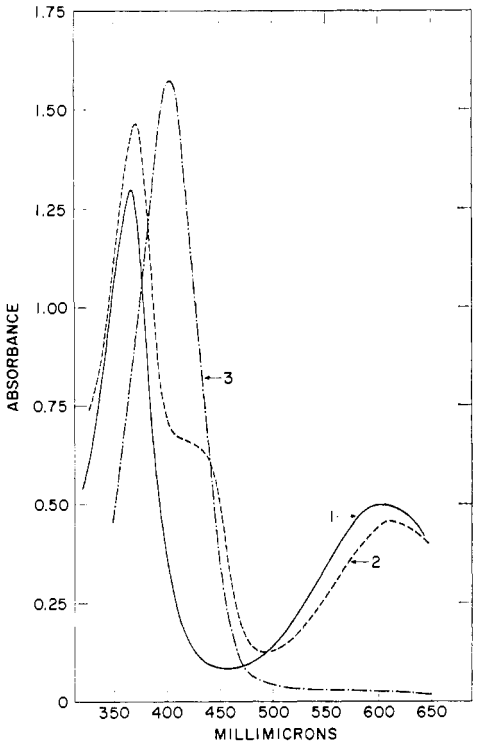

FIGURE 1: Electronic spectra of PCB (1), PCB heated to 195° under vacuum for 2 min (2), and the yellow material sublimed under vacuum by heating PCB with a luminous flame (3). Sublimate was condensed on a cold finger cooled with powdered Dry Ice and placed about 0.5 mm from the heated sample. All spectra were run in chloroform containing about 0.75% ethanol.

Results and Discussion

Structure and Hydrogen Exchange of Phycocyanobilin

Visible Absorption Spectra and Chromatographic Behavior of PCB and Some of Its Reaction Products. Table I presents a summary of the absorption spectra of PCB in freshly destabilized chloroform and in chloroform containing varying amounts of ethanol. These data indicate that, in terms of absorption spectra, the phycocyanobilin isolated by refluxing with methanol is the same as phycobilin 630 isolated by Ó hEocha (1963) by hydrochloric acid treatment. Table II lists a number of absorption maxima obtained with phycocyanin and

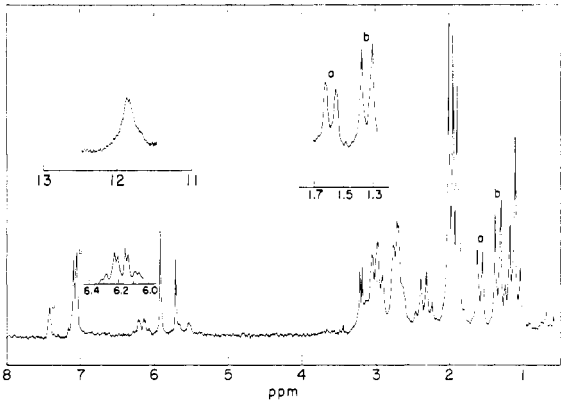

FIGURE 2: The 100-Hz proton magnetic resonance spectrum of phycocyanobilin dissolved in pyridine-d_5. The assignments of the lines are summarized in Table III. The doublets at a (1.58 ppm) and b (1.34 ppm) are on an expanded scale to show an unresolved small splitting of doublet *a*. The multiplet at 6.17 ppm is also shown on an expanded scale and enhanced by repetitive scanning. The rapidly exchangeable protons appear at 11.9 ppm. The lines marked s are solvent lines.

TABLE III: Chemical Shifts and Proton Magnetic Resonance Assignments for Phycocyanobilin in Pyridine-d_5 (relative to internal HMS) and 0.1 M TFA (relative to external HMS).

Chemical Shift (ppm)			Rel Area (nearest integral	
Pyridine	TFA	$\|J\|$, Hz (±0.1)	value)	Assignment
1.11	0.99	7.5, triplet	4[a]	CH_3CH_2[b]
1.34	1.37	7.5, doublet	3	CH_3CH[c]
1.58	1.84	7.1, doublet	3	$CH_3CH{=}$[d]
1.89	2.02			
1.95	2.06	Singlets	9	CH_3 ring
2.01	2.06			
2.34	2.32	7.6, quartet	2	CH_3CH_2[b]
2.70	2.65		4	
		Multiplets		CH_2CH_2COOH
2.97	3.04		4	
3.2[e]	3.43	Multiplet		CH_3CH[c]
3.17				
3.20		Singlets		CH_3O
5.71	5.97	Singlet	1	CH=, bridge *c*
5.92	6.42	Singlet	1	CH=, bridge *a*
6.17	6.62	7.2, 2.1 quartet of doublets	1	$CH_3CH{=}$[d]
7.09	7.37	Singlet	1	CH=, bridge *b*
11.9[f]		Singlet	5	NH and COOH

[a] High value believed due to impurity, perhaps some ethyl ester (see Figure 4). [b–d] The spin–spin interactions in these pairs of groups confirmed by decoupling experiments. [e] The presence of this proton deduced from the fact that decoupling irradiation at this frequency causes collapse of the doublet at 1.34 ppm to a singlet. [f] Concentration dependent. Probably averages with traces of water in the solvent.

phycocyanobilin in a variety of aqueous systems. Of particular interest is the pH dependence of the electronic spectra. The shift in the absorption maximum of phycocyanobilin from 600 mμ at pH 8.1 to 635 mμ at pH 5.3 is consistent with the presence of a pyrrolenine (N=) group, as reported by Ó Carra and Ó hEocha (1966). Because native phycocyanin, however, shows no such marked pH dependence (Hattori *et al.*, 1965b), the possibility still remains that the pyrrolenine grouping is an artifact in the product as isolated; more probably, the prosthetic group is buried or hydrogen bonded (or both) in the native protein as suggested by by Ó hEocha (1963). Table II shows that in 7 M urea solution, the pH dependence of the visible absorption spectrum of phycocyanin is similar to that of the isolated phycocyanobilin and strongly suggests that the pyrrolenine group must occur in native PCB.

Figure 1 shows the result of heating solid PCB to a temperature of 195°. As compared to the original PCB, the heated material shows a shift to longer wavelength in the red band and the appearance of a pronounced shoulder at about 420 mμ. When PCB is heated in a sublimation apparatus under vacuum, a yellow material deposits on the cold finger; the absorption spectrum of this sublimate is also given in Figure 1. Chromatographic analysis of heated material (solvent C, Adsorbosil 3) showed a green spot (R_F 0.56) and a yellow spot (R_F 0.72, white under ultraviolet light) in addition to the original PCB (R_F 0.35). A chromatographically identica yellow substance is formed by the reduction of PCB with sodium hydrosulfite in acetic acid.[4] These experiments provide strong presumptive evidence for the disproportionation of the free acid on heating and may be pertinent to the interpretation of mass spectral data. Jackson *et al.* (1966) have previously observed an apparent disproportionation of stercobilin under conditions of mass spectral analysis.

Phycocyanobilin can easily be converted into a purple pigment (λ_{max} 560 and 322 mμ, pyridine solution) by treatment with weak aqueous base. The extreme ease of reaction is probably the result of the acidity of the imide hydrogen of ring D, and the purple product is most probably violinoid, but has not as yet been fully characterized. The purple pigment does not give a transient blue color in alkali, as do phycoerythrobilin and aplysioviolin (Rüdiger *et al.*, 1967), probably because of the presence of an ethyl group rather than a vinyl group at position 8 and an absence of the concomitant tautomerism (Nicholson, 1967).

Proton Magnetic Resonance Analysis. Figure 2 shows the proton magnetic resonance spectrum of phyco-

[4] Under these reaction conditions, a second yellow pigment is also formed in somewhat greater amount. No other colored materials are formed by this reduction.

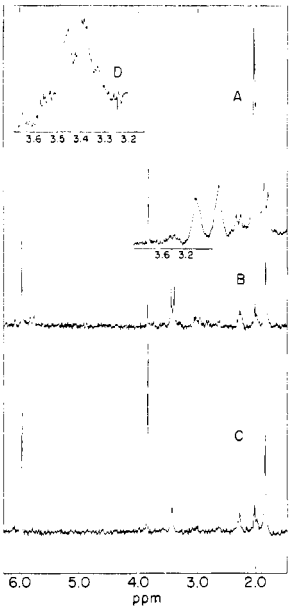

FIGURE 3: Spectral studies. (A) A portion of the spectrum of PCB in trifluoroacetic acid. A broad multiplet can be seen at 3.43 ppm. This multiplet is shown at D, enhanced by repetitive scanning. A quartet structure is obvious, but the smaller splittings are unresolved. (B) A portion of the spectrum of DPCB in trifluoroacetic acid. The spectrum was recorded 1 hr after solution and shows a little conversion of the methoxy group at 3.4 ppm into free CH_3OH at 3.84 ppm. Spectrum C is the same as B, but several weeks later. The ester linkages are completely hydrolyzed and a new line has grown in by exchange, the proton at position 1, ring A (see Figure 7). Because the spin coupling of this proton is now to deuterium atoms, the line appears as a broad singlet.

cyanobilin isolated from phycocyanin by refluxing with methanol. The interpretation of this spectrum, as well as the spectrum in trifluoroacetic acid solution, is summarized in Table III. In both solvents, spin–spin interactions were confirmed by decoupling experiments. The two small singlets at 3.17 and 3.20 ppm are due to a small amount of methyl ester probably formed during the isolation procedure. These lines are absent in PCB isolated by refluxing with ethanol. However, PCB isolated by refluxing with ethanol contains some ethyl ester, and the ethyl group confuses the interpretation of the upfield portion of the PCB spectrum. Traces of ethanol in the chloroform used in the preparative procedure also form some ethyl ester that can contribute extraneous small lines.

In pyridine, the methoxy lines obscure a multiplet with a chemical shift of *ca.* 3.2 ppm. The presence of an additional proton resonance at this point can be deduced from the fact that decoupling irradiation at this frequency causes collapse of the doublet at 1.34 ppm to a singlet. A more direct result can be obtained by analysis of PCB dissolved in trifluoroacetic acid. Upon standing, the ester undergoes hydrolysis, and a methanol line (CH_3OH) can be seen to grow in at 3.84 ppm. As shown in Figure 3A, the origin of the multiplet at 3.43 ppm (TFA) can now be deduced. Enhancement of these lines by repetitive scanning yields the quartet of lines shown in Figure 3D. The lines are broad, indicating additional unresolved splittings, and decoupling experiments indicate that this proton is not only coupled to the protons at 1.34 ppm, $|J| = 7.5$ Hz, and that at 6.17 ppm, $|J| = 2.1$ Hz, but is also coupled to the ethylidene methyl doublet at 1.58 ppm. The expanded spectrum in Figure 2 (lines a and b) shows the incipient splitting of this doublet at 1.58 ppm.

This system involves spin–spin coupling through five bonds. The geometry of this system should then be similar to the fixed geometry of similar systems in which spin–spin coupling through five bonds is observed (Freeman, 1963). In these systems, a double bond is located between two pairs of single bonds. The methyl protons of the ethylidene group should then form such an arrangement with the proton at position 1 ($\delta = 3.2$ ppm). The structures of Figure 7 satisfy this condition.

The assignment of the methine resonances to particular bridge positions can be deduced by comparison of the data of this work with that of Cole *et al.* (1967). Table IV compares the chemical shifts of the methine

TABLE IV: Chemical Shifts of Methine Bridge Protons in Various Pigments.

Compound	δ of Methine Proton		
	a	*b*	*c*
Phycocyanobilin, free acid	5.92	7.09	5.71
Phycocyanobilin, diester[a]	5.90	6.84	5.70
Mesobiliverdin, diester[a]	5.76	6.93	5.71

[a] Data of Cole *et al.* (1967).

protons in the free PCB acid with the dimethyl esters of phycocyanobilin and mesobiliverdin. The chemical shift of the proton at bridge *c* is invariant, as is to be expected from the molecular structure at this position. At bridge *a*, the same chemical shift is expected (and observed) in the free acid and diester of PCB. At bridge *b*, differences among all three compounds are expected. These arguments lead to the methine bridge assignments of Table III.

The available data (this work; Crespi *et al.*, 1967; Cole *et al.*, 1967; Rüdiger *et al.*, 1967) enable one to deduce the structure of the side chains of phycocyanobilin. A type IX arrangement of these side chains is indicated. These data, however, do not establish the position of the ethylidene group (position 2 or 8) unequivocally and give no indication of the relative importance of the possible prototropic forms of PCB. The exchange data described below shed some light on these problems.

Exchange Behavior of Phycocyanobilin. When fully deuterated phycocyanin is refluxed with ordinary methanol, phycocyanobilin (DPCB) containing ^1H only at exchangeable positions or at sites of chemical reaction can be isolated. Proton magnetic resonance examination of such a product (dissolved in pyridine-d_5) showed

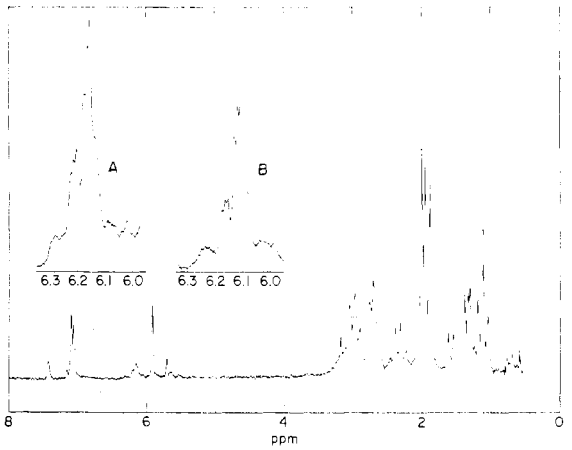

FIGURE 4: The proton magnetic resonance spectrum of PCB first isolated with CH_3OH, then refluxed with neutral CH_3OD. The introduction of deuterium into the ethylidene methyl group markedly changes the normal quartet structure at 6.17 ppm (inset A). Inset B is the same as inset A, but under deuterium decoupling. The 2.1-Hz splitting, because of coupling to the proton at position 1, can be observed in inset B. The hydrogen at position 1 is partially exchanged for deuterium, as the methyl group at position 1 (1.33 ppm) shows both doublet (J_{HH}) and apparently singlet (J_{HD}) structure. The introduction of a vicinal deuterium atom has caused a diamagnetic shift of 0.72 ± 0.05 Hz in the chemical shift of this methyl group.

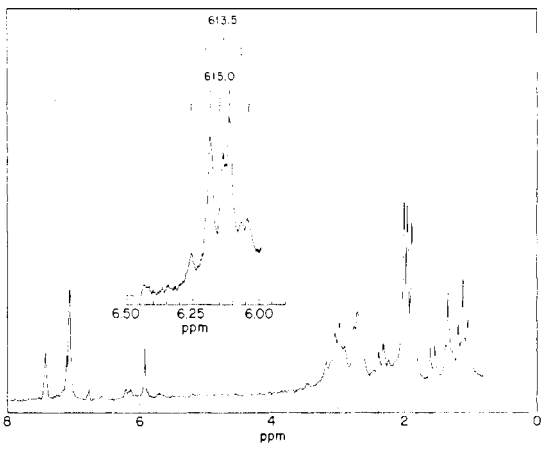

FIGURE 5: The proton magnetic resonance spectrum of PCB first isolated with CH_3OH, then refluxed with slightly acid CH_3OD. The downfield multiplet seems clearly composed of a quartet at 615 Hz and a triplet at 613.5 Hz, indicating the presence of $CH_3CH{=}$ and $CH_2DCH{=}$. The diamagnetic shift of 1.5 Hz due to the vicinal deuterium atom is unusually large. The resonance due to the methyl group at position 1 (nominally at 1.33 ppm) is now almost completely devoid of doublet structure, indicating almost complete exchange of the proton at position 1 for deuterium.

singlet lines of near-equal intensity at 1.58 and 5.71 ppm and two small methoxy singlets at 3.17 and 3.20 ppm. (DPCB isolated by refluxing fully deuterated phycocyanin with CH_3OD shows only the two methoxy lines.) When fully deuterated phycocyanin is refluxed with ordinary ethanol, the product DPCB gives proton resonance lines at 1.58 and 5.71 ppm, but we also see a weak line at 3.17 ppm. This proton at 3.17 ppm has been assigned tentatively to position 1 (see Table II). To account for its exchangeability, it must be α to a carbonyl group. The line at 1.58 ppm corresponds to the ethylidene methyl group and the line at 5.71 ppm to the proton of the methine bridge *c*. Similarly, in Figure 3B we see a portion of the proton magnetic resonance spectrum of DPCB dissolved in trifluoroacetic acid. Again we see proton resonances that are assigned to the ethylidene methyl group, the proton at methine bridge *c*, and the methoxy singlets. After standing several weeks, the methoxy lines are observed to be at 3.84 ppm, presumably due to hydrolysis of the ester, and in their place there now appears a broad singlet. This new line has apparently grown in by exchange with the solvent and indicates that this particular exchange proceeds fairly readily in strong acid. However, the line corresponding to the ethylidene methyl group (1.84 ppm in TFA, external HMS) is unchanged in intensity, so that if this proton is introduced by exchange at this position, the process is not acid catalyzed.

The possibility that a proton is introduced into the ethylidene methyl group during the process of detachment from the apoprotein cannot be dismissed. By using the proton at the methine bridge *c* as an internal standard, we find 1.3 ± 0.3 protons introduced into DPCB prepared by refluxing deuterophycocyanin for 18 hr with ordinary methanol. In order to establish exchange at this position and at the other positions into which ^1H has been introduced in DPCB, we have refluxed ordinary (^1H) PCB with CH_3OD. In such an experiment, introduction of deuterium will result in a change in the multiplicity of some of the proton resonances. The result of such an experiment is shown in Figure 4. A calculated amount of dibasic sodium phosphate was added to the reaction mixture to give a neutral solution, and thus to mimic the usual isolation procedure. The proton magnetic resonance data may be interpreted as follows. (1) The proton at 5.71 ppm has been almost completely exchanged or, more probably, completely exchanged and partially back-exchanged during the isolation procedure; (2) the proton at 3.17 ppm has been partially exchanged, leading to the singlet at 1.33 ppm;[5] (3) deuterium has been introduced into the methyl group of the ethylidene group at position 2. The area of the doublet at 1.58 ppm is reduced, and the structure of the doublet of quartets at 6.17 ppm is strongly modified, as shown in Figure 4 (inset A). Inset B is the same region as inset A, but recorded under deuterium decoupling. These spectra are not easily analyzed, but they do indicate the presence of triplet structure and, therefore, a considerable admixture of CH_2D–CH in the ethylidene grouping.

In an experiment similar to the one just described, PCB (^1H) was refluxed with CH_3OD but with only one-tenth the amount of dibasic phosphate required to provide a neutral solution. This slightly acid solution of PCB was then refluxed overnight, and the PCB was

[5] The introduction of a deuterium atom at position 1 results in a much reduced coupling constant to the methyl group at this position, and also causes a slight diamagnetic shift in the resonance position of this methyl group.

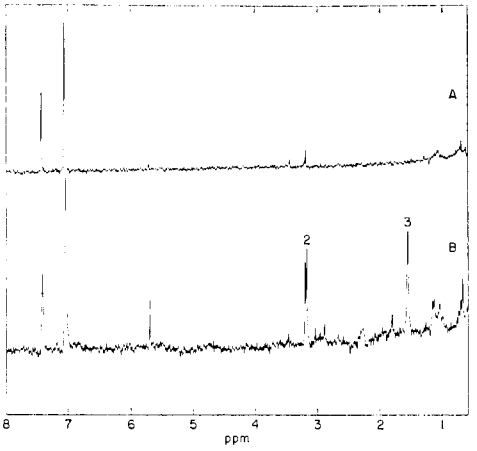

FIGURE 6: Proton magnetic resonance spectra. (A) In pyridine-d_5 of DPCB isolated by refluxing with CH_3OD. The only proton resonances are those of the solvent at low field, the small methoxy lines at 3.2 ppm, and a small amount of protons about 1 ppm. (B) DPCB isolated as in A and then refluxed for 18 hr with neutral CH_3OH. Clearly, the lines at 1.58 (3) and 5.71 ppm (1) are introduced by exchange.

reisolated for analysis.[6] This material gave the spectrum shown in Figure 5. The proton at position 1 has been almost completely exchanged in the more acid solution, as the methyl group at position 1 shows almost no doublet structure. The downfield multiplet shows up clearly as a mixture of triplet and quartet structures. The extent of exchange in the ethylidene group is not markedly different from that induced by refluxing in neutral solution, and is probably somewhat less. Finally, when DPCB isolated with CH_3OD is refluxed with neutral CH_3OH, the spectrum shown in Figure 6 is obtained. Lines at 1.58 and 5.71 ppm have been introduced by exchange. The data show, then, that phycocyanobilin contains three types of carbon-bound hydrogen that are exchangeable: (1) the proton at position 1; (2) the ethylidene methyl group; and (3) the bridge methine proton at bridge *c*.

Structure of Phycocyanobilin. The data reported here are best fit by structure A of Figure 7. The base-catalyzed exchange of the ethylidene methyl group can be explained in terms of the ionic form (Figure 7E). The exchangeability of the proton whose resonance line is at 3.17 ppm establishes unequivocally that the ethylidene group is at position 2, as there is spin–spin coupling (2.1 Hz) between the ethylidene methine proton and the proton at position 1. Placement of the ethylidene group at position 2 allows the formation of the apparently minor prototropic forms B–D with only slight changes in the extent of conjugation. The big difference in chemical shift of the ethylidene methyl group between pyridine and trifluoroacetic acid is accounted for by structure A, as the pyrrolenine nitrogen is on a path of cross-conjugation to the ethylidene group and its protonation will easily be felt at the ethylidene group.

[6] In this experiment, only about half the original amount of PCB was recovered. Large amounts of a green side product were formed.

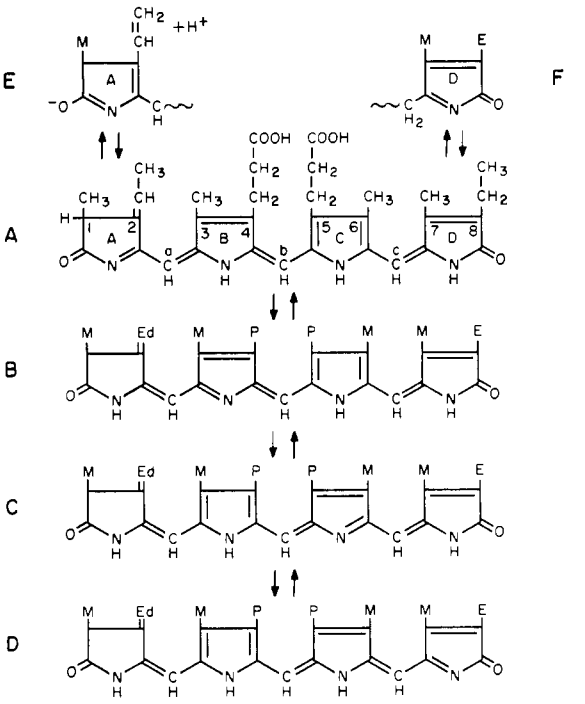

FIGURE 7: Structure proposed for phycocyanobilin. Structure A fits the proton magnetic resonance structural and exchange studies and the mass spectral data. The structures B, C, D, and F are possible prototropic forms of A, and the formation of the ion E explains the exchange at the ethylidene group.

The recently reported high-resolution mass spectral data (Crespi *et al.*, 1967) showed a parent ion at mass 588.293 that required a formula $C_{33}H_{40}N_4O_6$. At high ionizing voltages, an ion is observed at *m/e* 586 of about 40% the intensity of *m/e* 588. The disproportionation of phycocyanobilin on heating which we have observed makes it possible that the ion *m/e* 588.293 represents PCB plus 2H. Thus, an ambiguity still remains, and the mass spectral behavior of the free acid of phycocyanobilin requires further study. At present, the mass spectral data are compatible with a molecular weight of either 586 or 588. The chemical and spectral properties of the bilin are accounted for more easily by structure A of Figure 7. But the possibility that the bilin has a lower oxidation state cannot be excluded with the information now at hand.

Attachment of Phycocyanobilin in Phycocyanin

Amount of PCB in Phycocyanin. The PCB content of phycocyanin has been subject to considerable uncertainty. Ó hEocha (1966) reported the finding of 4.7% PCB in C-phycocyanin. This value corresponds to 3.7 prosthetic groups/phycocyanin monomer (mol wt 45,000). Other workers have found values of 3.6% (Lemberg and Legge, 1949), 4.0% (Clendenning, 1954),[7] and 5.6% (Brody and Brody, 1961), corresponding, respectively, to 2.8, 4.0, and 4.3 prosthetic groups per phycocyanin monomer unit.

In this work, we have taken a known amount of

[7] Cited in Ó hEocha (1963).

TABLE V: The Effect of Proteolytic Digestion on the Visible Spectrum of Phycocyanin.[a]

Enzymes Used	Initial		At 10 min		At 35 min		At 3 hr	
	A_{620}	A_{350}	A_{600}	A_{360}	A_{600}	A_{360}	A_{600}	A_{360}
Trypsin and chymotrypsin	1.53	0.26	0.37	0.70	0.32	0.75	0.30	0.54
Trypsin, chymotrypsin, and carboxypeptidase A	1.50	0.25	0.41	0.67	0.30	0.61		

[a] Experiments run in 0.1 M tricine buffer (pH 8.25) at 25°. A large excess of each enzyme was used. The concentration of phycocyanin in each case was 0.021%, based on an absorptivity of 7 (Hattori *et al.*, 1965a,b).

native phycocyanin and subjected it to proteolytic digestion. During the course of digestion the intensity of the blue band (A_{360}) is enhanced by a factor of 2.7. This is a maximum value, as PCB is slowly converted into violin at alkaline pH and A_{360} slowly drops. We then make the assumption that the absorbancy of the blue band in the digested material is essentially the same as the absorbancy of the blue band of free PCB in the same solvent. Table V illustrates a typical set of data. Purified PCB in 0.02 M tricine buffer (pH 8.2) gives $E^{1\%}_{1\,cm}$ 670 at A_{360} and $E^{1\%}_{1\,cm}$ 200 at A_{600}. From these data we calculate a content of 4.0% PCB in native C-phycocyanin. From this number a minimum molecular weight for phycocyanin of 14,500 can be calculated. Since the monomer unit has a molecular weight of about 45,000, three prosthetic groups per monomer must be present.[8] A value of 4.0% for the bilin content of phycocyanin is also consistent with the material balance during the preparative procedure. Based on this PCB content of phycocyanin, yields of purified material range from 40 to 50%. Spectral analyses (in chloroethanol[9]) of residual protein, after methanol extraction, indicated about 20% of prosthetic group remaining in the protein. Typically, for phycocyanin from both *P. luridum* and *S. lividus*, the ratio of A_{350} to corrected A_{280} was 2.5 for native phycocyanin and 0.5 for the methanol-extracted material. If purified PCB is refluxed overnight in methanol and again isolated, the yield is 75% of the starting material. Thus, approximately all the PCB can be accounted for. This result is of some importance because it indicates a similar covalent attachment for all three PCB molecules in the monomer unit.

Gel Filtration Experiments. In an attempt to learn something of the amino acid environment of the bilin in phycocyanin, we have digested phycocyanin with various combinations of trypsin, α-chymotrypsin, and carboxypeptidases A and B, and then fractionated the digests on a Sephadex G-15 column. Table VI indicates the enzymes employed. In Figure 8, we give an elution pattern typical of phycocyanin after digestion with trypsin, chymotrypsin, and carboxypeptidase A. (Inclusion of carboxypeptidase B gives an identical result.) The prosthetic group is eluted as a broad band beginning at about 40 ml and tailing to about 125 ml. Absorbance measurement in the ultraviolet indicates the presence of aromatic amino acids that peak at 50, 60, and 135 ml. Amino acid analysis and gel filtration with known amino acids show that at these elution volumes free phenylalanine, tyrosine, and tryptophan, respectively, are present.[10] The great bulk of the amino acids in the digest appears in the form of peptides at low elution volumes. It becomes possible, then, to analyze the fractions containing the bulk of the prosthetic group to see which amino acids are associated with the bilin. Since no blue color is extractable into chloroform upon acidification of the digest, at least one amino acid must still be linked to PCB after enzymic digestion. Since the

TABLE VI: The Retention of Blue Color from Various Phycocyanin Digests.

Digesting Enzymes	Retained on G-15
Trypsin and chymotrypsin	No
Chymotrypsin and carboxypeptidase A	No
Chymotrypsin, trypsin, and carboxypeptidase A	Yes
Chymotrypsin, trypsin, and carboxypeptidases A and B	Yes

[8] Berns *et al.* (1964) has reported a monomer molecular weight of 30,000 for C-phycocyanin. In this case there would be two PCB molecules per protein subunit.

[9] Calculations made on the basis of ratios of absorbance at 280 mμ (protein) to that at 350 mμ (PCB) before and after methanol extraction. The contribution of PCB to the absorption at 280 mμ was estimated to be 0.4 × A_{350} from measurement on free PCB. In the native protein, then, 50% of the absorbance at 280 mμ would be derived from the PCB moiety. Bannister (1954) calculated a value of 46% for the apoprotein contribution to A_{280} of phycocyanin.

[10] This experiment constitutes a satisfactory analysis for tryptophan, as no tryptophan can be found in the early fractions. Calculation indicates two molecules of tryptophan per phycocyanin monomer (mol wt 45,000), and this confirms the data of Cope *et al.* (1967).

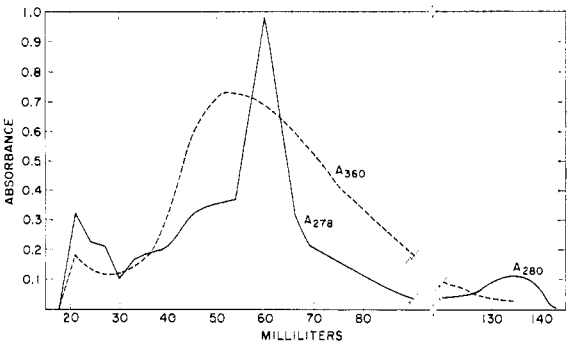

FIGURE 8: Elution patterns from Sephadex G-15 of phycocyanin after digestion with trypsin, chymotrypsin, and carboxypeptidase A. The prosthetic group was monitored at 360 mμ and the aromatic amino acids at 278 or 280 mμ. The peak at 50 ml is due to phenylalanine; at 60 ml, tyrosine; and at 135 ml, tryptophan.

molecular weight of PCB is about 600, only a small peptide can still be linked to the PCB. Free PCB is strongly adsorbed to Sephadex and does not elute under the conditions of these experiments.

Figure 9 shows the results of analysis of fractions high in PCB content. The concentrations of serine, glycine, alanine, aspartic acid, and leucine correlate with the PCB level, as measured by absorbancy. At 60 ml, all other neutral and acidic amino acids are at concentrations below 0.02 μmole/ml. Lysine and arginine are at much higher levels and do not fit the elution pattern of the bilin. The possibility of the attachment of the bilin to the protein *via* an amide link through lysine seems unlikely because of the difficulty of rupturing such a bond[11] with refluxing methanol. The fact that only very small amounts of methyl ester are formed during the detachment of PCB with methanol[12] would indicate that under our isolation conditions lysis is base catalyzed (or that PCB is liberated indirectly *via* a presently unknown mode of transesterification). However, the solution used for detachment of PCB is neutral. One may postulate, however, that microscopic regions about dissolved protein molecules are basic due to the high arginine content of phycocyanin. Amino acid analysis of the protein remaining after removal of PCB shows that the serine and the other amino acid content are essentially the same as for native phycocyanin. No new peaks were observed, so it is most probable that no chemically modified amino acids are present, except, of course, those that would revert to normal form on acid hydrolysis.

Mode of Attachment of PCB. Phycocyanobilin appears to be covalently linked to apoprotein through an

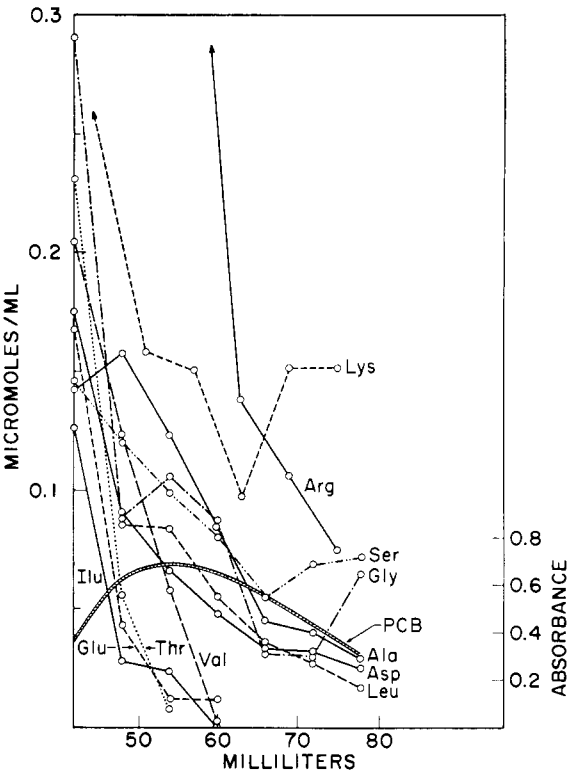

FIGURE 9: A detail of the elution pattern (Sephadex G-15) from 50 to 80 ml of phycocyanin after digestion with trypsin, chymotrypsin, and carboxypeptidase A. The broad, hatched line shows the PCB level at these elution volumes, as monitored at 360 mμ. (An absorbance of 0.5 corresponds to about 0.1 μmole/ml of PCB.) These data indicate that PCB may be attached to a peptide composed of serine, glycine, alanine, aspartic acid, and leucine.

ester bond[13] and also well buried in the protein moiety. The lack of a marked pH dependence of color in native phycocyanin (Ó hEocha, 1963; Hattori *et al.*, 1965b) indicates that the bilin is buried and perhaps hydrogen or ionic bonded, or both, since there are three prosthetic groups per monomer, through the pyrrolenine nitrogen. The color of isolated PCB shows a strong pH dependence (Table II). The behavior of native phycocyanin upon reduction with borohydride supports the view that the bilin is buried. In aqueous 0.1 M Tris buffer (pH 7.5–8.5), proteolytic digests of phycocyanin are completely reduced by potassium borohydride to a yellow material (λ_{max} 415 mμ) in 40 min. Under similar conditions native phycocyanin is only about half converted into the yellow product and the reaction is continuing only very slowly. At higher ionic strengths, reduction of the native phycocyanin proceeds even more slowly. High ionic strength favors formation of phycocyanin hexamers and, presumably, a tighter structure, so the bilin would become more inaccessible under this condition. It is interesting to note that at about 40 ml the peptides that are eluted are extremely rich in leucine, isoleucine, and valine. It is quite pos-

[11] A consideration of the specificities of the enzymes involved in the digestion procedure also indicates that lysine is not attached to PCB. The necessity of using trypsin to obtain small blue peptide fragments indicates that a lysine or arginine residue is just up-chain from PCB.

[12] In the PCB isolated by refluxing with methanol with 10% water or with "anhydrous" methanol (~0.5% water) one observes from 5 to 25% total monoesters. Ó Carra and Ó hEocha (1966) also show that the methanolic liberation of bilin from apoprotein does not involve extensive methylation of the propionic acid side chains of the phycobilin.

[13] Recent experiments by Siegelman *et al.* (1967) show that this bond can be broken and the bilin liberated by the enzyme preparation Nagarse.

sible, then, that phycocyanobilin, even though a very large molecule, is enveloped in hydrocarbon-like regions of the protein.

Relevance to Structure of Phytochrome. Prima facia, phycocyanobilin is linked to the protein moiety through a serine side chain. We have no assurance, however, that the presence of serine in a peptide linked to PCB is not fortuitous, for the explanation of the methanolysis is not compelling. In considering alternate possibilities, one must include the mode of attachment illustrated by Figure 10 (red). It is possible that in biliproteins, such as phycocyanin, allophycocyanin, phycoerythrin, and phytochrome, the bilin is covalently attached to the peptide backbone through an ethylidene group. Detachment of the bilin from apoprotein by means of hot, neutral methanol, ethanol, and other alcohols could then proceed by simple transesterification with the formation of an ester of aspartic or glutamic acid and an OH group at the nominal carbonyl carbon of the peptide bond. Rearrangement would then lead to expulsion of PCB and the formation of a normal peptide bond.[14]

This mode of attachment of bilin to apoprotein offers an interesting model for the photochromic response of phytochrome. In phytochrome, coplanarity between the bilin and the involved C–N bond of the peptide bond could lead, upon absorption of light, to a charge-transfer state such as shown in Figure 10 (far red). The formation of similar photoreversible charge-transfer states is well known in spirans (Dessauer and Paris, 1963). Formation of a charge-transfer state as shown in Figure 10 should not be demanding energetically, as the exchange data for PCB presented here require a considerable contribution of a related ionic form to the structure of phycocyanobilin. The driving force for the reaction would come from the energy of the absorbed light and from the formation of a very stable free carboxyl group. The model can easily fit the photochemical measurements on phytochrome made by Linschitz and Kasche (1967). Upon absorption of 664-mμ light, P_r would go into a transition state (λ_{max} 695 mμ) and then into a metastable state characterized by λ_{max} 700 mμ. The slower changes leading to the final form, P_{fr} (λ_{max} 724 mμ), correspond to conformational changes in the protein. The reverse reactions would be rapid throughout, as observed by Linschitz and Kasche (1967), as optical decay to the P_r chromophore would not be dependent upon protein conformation.

Not only would the scheme of Figure 10 account for the red $\rightleftharpoons$ far red intraconversion in phytochrome, but the peptide bond to which the bilin is attached would act as a transducer for the conversion of the photochromic response into an allosteric response, as the newly formed C=N bond will be considerably shorter than a C–N bond. Conversions between red and far-red states would lead to backbone reflexes that would manifest themselves in conformational intraconversions

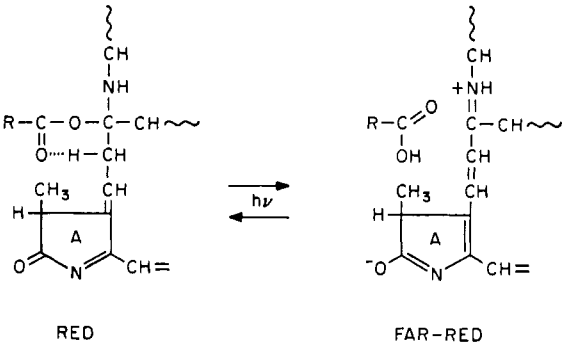

FIGURE 10: The structure labeled red represents a possible mode of attachment of phycocyanobilin to apoprotein. The nominal ethylidene group on ring A of PCB is linked to the carbon atom of a nominal carbonyl group of a peptide bond. No photochromism is observed in phycocyanin, but if the geometry between the bilin and the peptide bond were suitable, the absorption of red light would drive the red form into the far-red charge-transfer state. Upon absorption of far-red light, the charge-transfer state would revert to the red structure. This scheme could form the chemical basis for the photochromic responses of phytochrome. The hydrogen bond indicated in the red form is of a type recently suggested by Krimm (1967).

in the protein. Figure 10 thus seems a suitable first working model for the photoallosteric properties of phytochrome.

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[14] The data concerning exchange at the ethylidene group are not quantitative and cannot be taken to preclude this mode of attachment of PCB.

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The Chemical Composition of a Crystalline Bacteriochlorophyll–Protein Complex Isolated from the Green Bacterium, *Chloropseudomonas ethylicum**

J. P. Thornber and J. M. Olson

ABSTRACT: The amino acid composition and N-terminal residue of the bacteriochlorophyll–protein complex have been determined. The results reveal that the complex is very probably made up of four identical subunits, each of which contain 5 moles of bacteriochlorophyll. The molecular weight of such a subunit is 37,940; using this value and some previously published results the following physical properties of the complex have been calculated. The bacteriochlorophyll–protein concentration in the crystalline state is 0.43 g/cm^3; $\bar{V}$ = 0.764 ± 0.014 cm^3/g, molecular weight = (1.52 ± 0.04) × 10^5, molecular volume = (1.93 ± 0.08) × 10^5 Å^3; the specific volume of bacteriochlorophyll in the complex is about the same as that of crystalline chlorophyll a. The complex has been examined for the presence of components other than protein and chlorophyll; there was no other pigment or lipid but a trace of carbohydrate material was observed. Attempts to isolate a bacteriochlorophyll-peptide(s) by tryptic digestion of the denatured complex were not successful; the protein was removed from the pigment during proteolysis. The composition and properties of the complex show some striking similarities to those of a chlorophyll a containing protein of spinach beet.

The water-soluble bacteriochlorophyll–protein (Bchl-P)[1] complex of the green photosynthetic bacterium *Chloropseudomonas ethylicum* was isolated by Olson and Romano (1962). Olson (1966) reported that the complex had a molecular weight of (1.67 ± 0.17) × 10^5 g/mole and contained ~20 Bchl molecules. The biological function of the complex in the transfer of excitation energy has been demonstrated (Olson and Sybesma, 1963; Sybesma and Olson, 1963; Sybesma and Vredenberg, 1963) and there is some indication that *in vivo* the complex is associated with those molecules that take part in the primary photochemical reaction (Olson, 1964). An ellipsoidal model of the Bchl-P macromolecule *in vitro* is proposed (Olson *et al.*, 1968a) with a radial array of chromophore sites inside the ellipsoid (Olson *et al.*, 1968b). A detailed chemical analysis of the complex is reported in the present paper as a first step in elucidating the nature of chlorophyll–protein interactions and in understanding the nature of primary photosynthetic processes.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Bchl-P, bacteriochlorophyll–protein complex; Chl a, chlorophyll a.