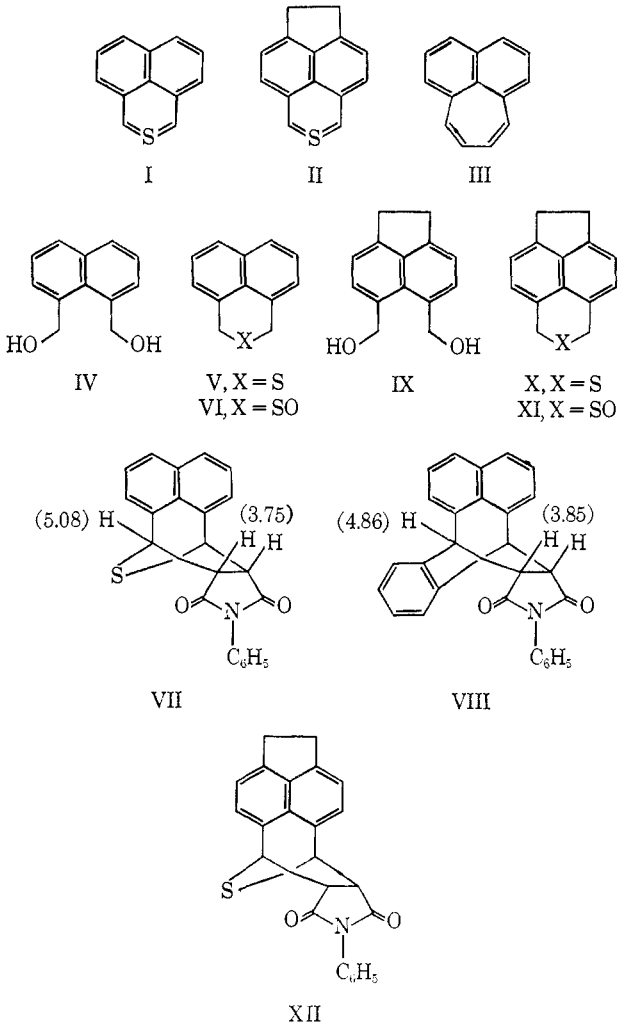

pleiadene adduct VIII.[7] Molecular models indicate deshielding of these protons in both VII[12] and VIII.

Treatment of 5,6-bis(hydroxymethyl)acenaphthene (IX)[13] with phosphorus pentasulfide in carbon disulfide gave in 80% yield sulfide X, mp 175°. On oxidation with sodium periodate, X afforded the corresponding sulfoxide XI, mp 292° dec, in 70% yield. The reaction of sulfoxide XI with refluxing acetic anhydride again was found to be complicated. However, when XI was allowed to react with N-phenylmaleimide under similar conditions a crystalline adduct XII, mp 342–357°, was isolated in 45% yield. Adduct XII was shown to have the *exo* structure indicated on the basis of its nmr spectrum, which closely resembled that of adducts VII and VIII.

Acknowledgments. This work was supported by a Frederick Gardner Cottrell grant from the Research Corporation and by the Petroleum Research Fund (Grant No. 756-G). We wish to thank Mr. Glenn P. Happ of the Eastman Kodak Company for determination of the mass spectra.

(12) Other features of the nmr spectrum of VII include the bridgehead protons at δ 5.08 and a broad band of eleven aromatic protons in the δ 7.20–7.81 region.

(13) L. A. Carpino and S. Gowecke, *J. Org. Chem.*, 29, 2824 (1964).

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Structure of Phycocyanobilin[1]

Sir:

The structure of the chromophore of the important photosynthetic pigment phycocyanin, present in blue-green algae, has long been of concern. Lemberg[2] described it as a linear tetrapyrrole, but much subsequent work[3] did not establish a structure. A major obstacle was the lack of a suitable preparative procedure for detaching the chromophore, without extensive alteration, from the protein moiety of the pigment. Recently Fujita and Hattori[4] found that refluxing phycocyanin, or even whole blue-green algae, with methanol containing ascorbic acid liberated the chromophore in good yield. Ó Carra and Ó hEocha[5] subsequently showed that the ascorbic acid was not essential to the liberation of the chromophore.[6]

C-Phycocyanin, isolated from *Phormidium luridum* or *Synechococcus lividus*,[7] is purified by standard methods.[8] After dialysis against 0.01 *M* phosphate buffer, pH 6.9, a 2–6% solution of the protein (2–4 g of protein) is diluted with methanol to 90% methanol and refluxed overnight. The blue solution is then centrifuged, evaporated to 200–300 ml, centrifuged, and extracted with petroleum ether (bp 20–40°). Then, 200 ml of freshly destabilized chloroform is added, and the mixture is acidified with glacial acetic acid and quickly washed three times with copious amounts of salt water. The blue chloroform solution is evaporated to dryness (at 30°). The residue is dissolved in 8–10 ml of chloroform containing 2.5% methanol, filtered, and then dried by rotary evaporation at 25°. The residue is taken up in about 4 ml of chloroform–methanol, precipitated by addition of seven volumes of petroleum ether, and washed with petroleum ether. Yields are 40% of the calculated on the assumption that the chromophore constitutes 5% (w/w) of the phycocyanin.

On mass spectrometric examination, phycocyanobilin shows a parent ion at *m/e* 588.293 ± 0.003. $C_{33}H_{40}N_4O_6$ requires 588.2948. This is the only observable ion at low ionizing voltages. The fragmentation pattern of phycocyanobilin[9] was similar to that of bilirubin,[10] showing a relatively weak parent ion and

(1) Based on work performed under the auspices of the U. S. Atomic Energy Commission.

(2) For a review and references to the literature, see R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, pp 127, 145–149.

(3) *Cf.* C. Ó hEocha in "Chemistry and Biochemistry of Plant Pigments," T. W. Goodwin, Ed., Academic Press Inc., New York, N. Y., 1965, Chapter 6.

(4) Y. Fujita and A. Hattori, *J. Gen. Appl. Microbiol.* (Tokyo), 9, 253 (1963).

(5) P. Ó Carra and C. Ó hEocha, *Phytochemistry*, 5, 993 (1966).

(6) Ó Carra and Ó hEocha[5] concluded that the chromophore liberated by methanolysis of phycocyanin was an "artifact." However, we find the absorption spectra of the methanolysis product to be identical with that of chromophore liberated by the conventional concentrated hydrochloric acid method, taking into account the solvent and acidity dependence of the visible absorption spectra. We have every reason to suppose that the methanolysis and hydrochloric acid products are at least very similar if not identical. Our compound shows very strong absorption maxima at 365–370 mμ in all solvents. In tetrahydrofuran, λ_{max} 590 mμ; in $CHCl_3$, λ_{max} 638 mμ; in CH_3OH–5% concentrated HCl, λ_{max} 680 mμ.

(7) H. F. DaBoll, H. L. Crespi, and J. J. Katz, *Biotechnol. Bioeng.*, 4, 281 (1962).

(8) A. Hattori, H. L. Crespi, and J. J. Katz, *Biochemistry*, 4, 1213 (1965).

(9) Measured with A.E.I. MS-9 mass spectrometers. A direct inlet system at ~220°, 70 ev, and 100 μa trap current was used.

intense dipyrrolic and monopyrrolic fragment ions. The empirical formula and the fragmentation pattern are compatible with a mesobilirubinoid tetrapyrrole. Structures that are closely related to mesobiliverdin may be eliminated on the basis both of empirical formulas and fragmentation pattern. The base peak in the mass spectrum of dimethylmesobiliverdin was the parent ion at *m/e* 614, and this compound gave only low intensity dipyrrolic fragment ions.[11] In contrast, the base peak in the spectrum of phycocyanobilin corresponded to a dipyrrolic fragment ion *m/e* 288.1478; $C_{16}H_{20}N_2O_3$ requires 288.1474.

The results of nuclear magnetic resonance analysis are summarized in Table I. The ethylidene group deduced from the nmr data has very recently been reported by Rüdiger to occur in the bile pigment aplysioviolin extracted from the Mediterranean sea hare.[12]

Table I. Nmr[a] Assignments for Phycocyanobilin Based on Chemical Shifts in 0.1 *M* Pyridine-d_5 (Relative to Internal Hexamethylsiloxane (HMS)) and 0.1 *M* Trifluoroacetic acid (TFA) (Relative to External HMS)

Chemical shift, ppm			Relative area,	
Pyridine	TFA	$\|J\|$, cps (±0.1)	nearest integral value	Assignment
1.11	0.99	7.5, triplet	4[b]	CH_3CH_2[c]
1.34	1.37	7.5, doublet	3	CH_3CH[d]
1.58	1.84	7.1, doublet	3	CH_3CH=[e]
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[c]
2.70	2.65	Multiplets	4	–CH_2CH_2COOH
2.97	3.04		4	
3.2	3.43	Multiplet	1	CH_3CH[d]
3.17	...	Singlets	1	CH_3O
3.20	...			
5.71	5.97	Singlet	1	CH=
5.92	6.42	Singlet	1	CH=
6.17	6.62	7.2, 2.1, quartet of doublets	1	CH_3CH=[e]
7.09	7.37	Singlet	1	CH=
11.9[f]	...	Singlet	5	NH and COOH

[a] Nmr spectra determined with a Varian HA-100 spectrometer. [b] High value believed to be due to impurity. [c–e] The spin–spin interactions in these pairs of groups are confirmed by decoupling experiments. [f] Concentration dependent.

A structure consistent with all of our data is shown in Figure 1. The oxidation state assignment is based entirely on the mass spectrometric results and is made on the assumption that no thermally induced internal oxidation–reduction reactions occur in the phycocyanobilin in the mass spectrometer source. The ready exchange of one of the methine protons requires a mobile equilibrium between the keto (bilirubinoid) and enol forms of this bile pigment. The visible and nmr spectra indicate that the enol form predominates in solution. The distribution of side chains is arbitrary and is by analogy with the known bile pigments. It has not been proved that the substance isolated by our procedure actually occurs as such in nature, and the mode of attachment of the chromophore to the protein is still obscure.

(10) A. H. Jackson, G. W. Kenner, H. Budzikiewicz, C. Djerassi, and J. A. Wilson, *Tetrahedron*, 23, 632 (1967).

(11) R. C. Dougherty, to be published. We are indebted to Drs. H. W. Siegelman and John Cole for a sample of dimethylmesobiliverdin.

(12) W. Rüdiger, *Z. Physiol. Chem.*, 348, 129 (1967).

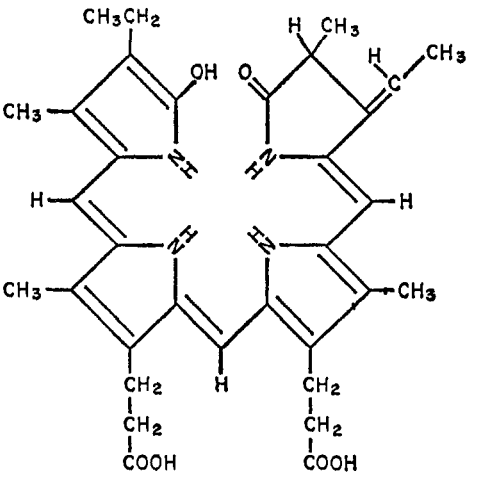

Figure 1. Proposed structure for phycocyanobilin.

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The Structure of Phycocyanobilin[1]

Sir:

C-Phycocyanin, a photosynthetically active algal protein, has a tetrapyrrolic chromophore, phycocyanobilin.[2] Evidence presented here shows the phycocyanobilin is 2-desethyl-2-ethylidene-1-protiomesobiliverdin, an isomer of mesobiliverdin (Figure 1).

Plectonema boryanum (Indiana University 594) cells (1200 g wet weight) were ground in a Waring blender with solid carbon dioxide, and the biliproteins after extractions with 0.1 *M* potassium phosphate buffer (pH 6) were separated from cell debris by centrifugation and precipitated with ammonium sulfate. C-Phycocyanin was chromatographed free of allophycocyanin on a Celite 545 column by ammonium sulfate gradient elution. The solution containing C-phycocyanin was made 1% with respect to trichloroacetic acid; the denatured protein (blue) was removed by centrifugation and washed successively with water (two 250-ml portions) and absolute methanol (four 250-ml portions). The denatured C-phycocyanin was boiled under reflux with absolute methanol (2.5 l.) with stirring for 16 hr, and the phycocyanobilin solution (blue) was filtered free of protein residue (38 g) and concentrated. Boron trifluoride in methanol (14%; 2 ml) was added to the methanolic pigment solution (2 ml), and the mixture was boiled under reflux. After 3 min the solution was cooled, and chloroform (10 ml) and water (100 ml)

(1) This work was performed at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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