The Structure of Phycoerythrobilin[1]

Sir:

C-Phycoerythrin, a photosynthetically active red biliprotein from blue-green algae, has a tetrapyrrole chromophore, phycoerythrobilin.[2] Evidence is presented to establish the structure of phycoerythrobilin as biladiene a,b (Figure 1). Phycoerythrobilin is isomeric with phycocyanobilin.[3,4]

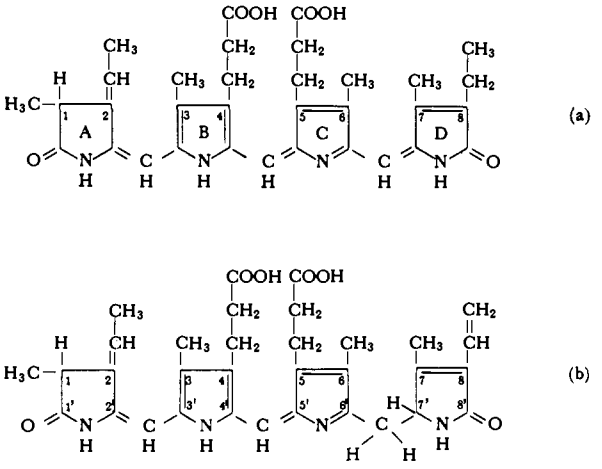


Figure 1. Structures of (a) phycocyanobilin and (b) phycoerythrobilin.

Phormidium persicinum cells (2000 g wet weight) were frozen and thawed in 0.1 *M* potassium phosphate buffer, pH 6, and then at pH 7. The extracted biliproteins were separated from the cellular residue by centrifugation and made to 5% saturation with ammonium sulfate. The C-phycoerythrin was denatured with 1% trichloroacetic acid and the precipitated protein washed at the centrifuge three times with water (3000 ml) and twice with methanol (1000 ml). The protein was suspended in 1500 ml of methanol with a Ten Broeck homogenizer and boiled under reflux with stirring for 16 hr. The red phycoerythrobilin solution was filtered from the residual protein and evaporated to a small volume (100 ml). The phycoerythrobilin free acid was esterified with diazomethane. Residual diazomethane was destroyed with a few drops of acetic acid; 150 ml of chloroform was added and then 1500 ml of water. The phycoerythrobilin dimethyl ester passed into the chloroform and was washed with water (three 750-ml portions), filtered through chloroform-soaked filter paper, and concentrated to 5-ml volume. The pigment was purified by thin layer chromatography on silica gel (Adsorbosil 5, Applied Science Laboratories, State College, Pa.) with carbon tetrachloride–methyl acetate (2:1 v/v). The principal red band was scraped from the plate, eluted with methanol, and rechromatographed in the same system. The methanol eluate of phycoerythrobilin dimethyl ester was evaporated to dryness, redissolved in a minimal volume of hot benzene, and precipitated by addition to cold petroleum ether (bp 30–60°). The precipitate was washed with petroleum ether and dried at ~55° *in vacuo* over paraffin chips. The pigment migrated as a single band on silica gel in benzene–ethanol (9:1.5 v/v) and ethylene dichloride–ethyl acetate (7:3 v/v). The yield of phycoerythrobilin dimethyl ester was 27 mg. Phycoerythrobilin dimethyl ester was also crystallized with difficulty from acetone–water as thin pink leaf-like crystals (mp 183–184° uncor). Phycoerythrobilin dimethyl ester is similar chromatographically and in electronic spectra to mesobilirhodin dimethyl ester[5] and mesobiliviolin dimethyl ester[5] (Table I).

Table I. Absorption Spectra Maxima of Phycoerythrobilin Dimethyl Ester, Mesobilirhodin Dimethyl Ester, and Mesobiliviolin Dimethyl Ester

	λ, nm				
	5% HCl–methanol		Satd $Zn(OAc)_2$–ethanol		
Phycoerythrobilin dimethyl ester[a]	591 (25.2)	326 (15.8)	603	557	336
Mesobilirhodin dimethyl ester	557	306	578	541	316
Mesobiliviolin dimethyl ester	556	324	626	576	334

[a] $\epsilon \times 10^{-3}$ values in parenthesis.

Table II. Nuclear Magnetic Resonance Assignments of Phycoerythrobilin Dimethyl Ester (~0.07 *M* in $CDCl_3$)[a]

Chemical shifts[b]	Rel intensity	Assignment	
6.64, singlet	1	Methine proton	—*CH*=
6.40, quartet of doublets	1	Ethylidene proton	CH_3CH=
6.25, multiplet	1	Vinyl	CH_2=*CH*—
5.80, singlet	1	Methine proton	=*CH*—
5.33, multiplet	2	Vinyl	CH_2=CH—
4.32, doublet	2	Methylene bridge	—CH_2—
3.63, 3.61 singlets	6	Methoxyl (2)	—OCH_3
3.24, triplet	1	7′ proton	
3.09, multiplet	1	Proton on saturated C_1	*H* | CH_3C—
2.90, triplet	4	β-Methylene (2) of propionic ester	—CH_2—
2.50, triplet	4	α-Methylene (2) of propionic ester	—CH_2—
2.01, 1.96 singlets	9	β-Methyl (3)	CH_3C=
1.89, doublet	3	Methyl of ethylidene	CH_3CH=
1.41, doublet	3	Methyl on saturated C_1	H | CH_3C—

[a] Nmr spectra kindly recorded by Mr. E. Gooden[6] with a Varian HA-100 nmr spectrometer. [b] Chemical shifts in parts per million (δ) from internal TMS.

The pigment was analyzed by nmr[6] (Table II). Assignment of the proton resonances was aided by comparison with phycocyanobilin dimethyl ester and

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

(2) P. O. Carra, C. Ó. hEocha, and D. M. Carroll, *Biochemistry*, 3, 1343 (1964).

(3) W. J. Cole, D. J. Chapman, and H. W. Siegelman, *J. Am. Chem. Soc.*, 89, 3643 (1967).

(4) D. J. Chapman, W. J. Cole, and H. W. Siegelman, *Biochim. Biophys. Acta*, in press.

(5) Prepared by ferric chloride oxidation of mesobilirubinogen (*cf.* C. H. Gray, A. Kulczycka, and D. C. Nicholson, *J. Chem. Soc.*, 2276 (1961) from bilirubin reduction (*cf.* C. J. Watson, *J. Biol. Chem.*, 200, 691 (1953)).

(6) We thank Dr. Hall of the U. S. Department of Agriculture for making the mass spectrometry and nmr facilities available

biliverdin dimethyl ester. The =NH resonances were imperceptible in the HA-100 spectrum.[3] These were assigned by elimination and from the observation[2] that phycoerythrobilin possesses only one pyrrolenine (—N=) nitrogen. The vinyl group and the unsaturated ring A were assigned by comparison with biliverdin dimethyl ester and phycocyanobilin dimethyl ester, respectively. Specifically there were no resonances attributable to the CH_3– and $-CH_2$– of an ethyl group, nor any attributable to a third methine bridge.

Mass spectral analyses[6,7] gave a strong base peak at *m/e* 614, requiring the empirical formula $C_{35}H_{42}N_4O_6$. The mass spectrum also showed an intense peak at *m/e* 492. This would be consistent with cleavage of the pyrrole (D) ring at the methylene bridge.

Validation of this structure is provided by two simple isomerizations. Treatment of phycoerythrobilin with 12 *N* HCl under N_2 at room temperature yields phycocyanobilin (identified as dimethyl ester by visible, ultraviolet, and infrared absorption spectra and mixed chromatography). Phycoerythrobilin, boiled in 1 *N* KOH in methanol under reflux for 15 min, yields mesobiliverdin (identified as dimethyl ester by visible, ultraviolet, and infrared absorption spectra and mixed chromatography).

Assignment of the 7′ proton to the pyrrole ring substituted with the vinyl group is based on the prototropic isomerizations to phycocyanobilin and mesobiliverdin and the strong *m/e* 492 peak.

The β substituents of rings A and D may be interchanged in the structure proposed, but the mass spectra pattern and the visible–ultraviolet absorption spectrum would support the conjugated system present in the structure outlined. Enzymatic cleavage of C-phycoerythrin with the enzyme Nagarse[8] yields phycoerythrobilin, providing further support that it is the native chromophore.

(7) Kindly recorded by Dr. J. Ruth in a CEC 21-110 mass spectrometer.

(8) H. W. Siegelman, D. J. Chapman, and W. J. Cole, *Arch. Biochem. Biophys.*, in press.

D. J. Chapman, W. J. Cole, H. W. Siegelman
Biology Department, Brookhaven National Laboratory
Upton, New York 11973
Received September 15, 1967

Molecular Geometry and Bonding in the Sydnone Ring

Sir:

The sydnones, which have been taken as prototypes of the general class of "mesoionic" compounds,[1] have thus far evaded a satisfactory formulation for their bonding.[2]

The most commonly written formula, I, whose implied aromaticity would account for the weak basicity of the sydnones[3] and for their benzenoid electronic spectra,[5,6] is difficult to reconcile with the high frequency (1768 cm^{-1}) and intensity of the carbonyl stretching band observed for the 3-alkylsydnones.[7]

(1) W. Baker and W. D. Ollis, *Quart. Rev.* (London), **11**, 15 (1957).
(2) F. H. C. Stewart, *Chem. Rev.*, **64**, 129 (1964).
(3) Sydnones crystallize from moderately concentrated mineral acid solutions.[4]
(4) H. U. Daeniker and J. Druey, *Helv. Chim. Acta*, **40**, 918 (1957).
(5) W. Baker, W. D. Ollis, and V. D. Poole, *J. Chem. Soc.*, 307 (1949).
(6) D. L. Hammick and D. J. Voaden, *ibid.*, 3303 (1961).

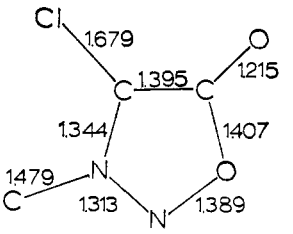


Figure 1.

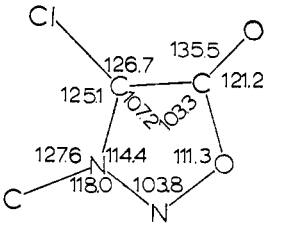
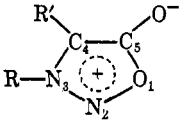


Figure 2.

A crystal structure determination of 3-*p*-bromophenylsydnone[8] indicated a molecular geometry which could not be interpreted in terms of structure I. It



I

was pointed out that the geometry of the sydnone ring might be influenced by a "charge transfer interaction" between the carbonyl oxygen and a neighboring bromine atom, and since the estimated standard deviations for bond distances were as high as 0.015 A, we felt that it was worthwhile to determine another sydnone structure. We chose for investigation 4,4′-dichloro-3,3′-ethylenebissydnone,[4] which crystallizes in the orthorhombic system, space group Pbca with eight molecules in the unit cell, thus permitting the determination of two crystallographically independent sydnone rings in the same crystal. The structure was determined by use of data collected with a Picker automatic diffractometer. The final *R* index for 1312 observed reflections was 0.042, and estimated standard deviations for bond lengths were about 0.004–0.005 A. Average bond distances and angles, corrected for anisotropic thermal motion effects,[9] are shown in Figures 1 and 2. The structure reported by Bärnighausen, *et al.*,[8] is in satisfactory agreement with ours, when due consideration is given to the standard deviations.

It is clear that all of the ring distances except that of the C–O bond are between single and double, and compare with, for example, the aromatic distances in benzene (1.397 A),[10] the C=N distance in pyridine (1.340 A),[11] the N=N distance in tetrazine (1.321 A),[12] and the N=O distance in 1,2,5-oxadiazole (1.380 A).[13]

(7) J. Fugger, J. M. Tien, and I. M. Hunsberger, *J. Am. Chem. Soc.*, **77**, 1843 (1955).
(8) H. Bärnighausen, F. Jellinek, J. Munnik, and A. Vos, *Acta Cryst.*, **16**, 471 (1963).
(9) D. W. J. Cruickshank, *ibid.*, **9**, 754 (1956).
(10) A. Langseth and B. P. Stoicheff, *Can. J. Phys.*, **34**, 350 (1956).
(11) B. Bak, L. Hansen-Nygaard, and J. Rastrup-Anderson, *J. Mol. Spectry.*, **2**, 361 (1958).
(12) F. Bertinotti, G. Giacomello, and A. M. Liquori, *Acta Cryst.*, **9**, 510 (1956).
(13) E. Saegebarth and A. P. Cox, *J. Chem. Phys.*, **43**, 166 (1965).