Cleavage of Phycocyanobilin from C-Phycocyanin

SEPARATION AND MASS SPECTRAL IDENTIFICATION OF THE PRODUCTS*

(Received for publication, October 31, 1975)

ROBERT J. BEUHLER, ROBERT C. PIERCE;‡ LEWIS FRIEDMAN, AND HAROLD W. SIEGELMAN

From the Departments of Biology and Chemistry, Brookhaven National Laboratory, Upton, New York 11973

Phycocyanobilin is the bile pigment chromophore of C-phycocyanin, the photosynthetically active biliprotein of blue-green and certain red algae. Several problems are encountered in attempts to characterize phycocyanobilin. Different forms of phycocyanobilin are obtained depending on cleavage conditions (1). Earlier studies indicated that only one form of phycocyanobilin had sufficient volatility for mass spectrometric analyses by conventional evaporation techniques (2). Discrepancies in molecular weight data attained by mass spectrometry were explained by a dehydrogenation of the molecule on esterification. The results of Crepsi et al. (3) and a subsequent study by Schram and Kroes (2) suggested that the molecular weight of the phycocyanobilin diacid was 588. Cole et al. (4) proposed that the phycocyanobilin diacid had a molecular weight of 586 based on a molecular weight determination of 614 for the phycocyanobilin dimethyl ester. They limited their investigations to the phycocyanobilin dimethyl ester because it was the principal component obtained on cleavage of C-phycocyanin and esterification, and was prepared in homogeneous form by thin layer chromatography and crystallization. Schram and Kroes (2) indicated that batch purification of phycocyanobilin diacid on silica gel was adequate because it was the only volatile component. They assumed that any impurities present had essentially no effect on the volatilization of the principal component. They confirmed the mass spectral analysis of phycocyanobilin dimethyl ester of Cole et al. (4), but did not seriously consider the alternative possibility of hydrogenation of the phycocyanobilin diacid in the mass spectrometer ion source.

This report presents detailed mass spectral analysis of the bile pigments cleaved from C-phycocyanin and their chemical interconversions. Volatility enhancement techniques developed for evaporating fragile natural products isolated from biological materials were successfully used to obtain proton transfer and electron impact mass spectra of molecular species that previously resisted evaporation and characterization by conventional electron impact mass spectrometry. Improved preparative and analytical separation techniques for bile pigments were developed utilizing high pressure liquid chromatographic methods.

EXPERIMENTAL PROCEDURE

Protein Purification—Blue-green algae Plectonema boryanum and Phormidium luridum were grown in mass culture (5). The biliprotein C-phycocyanin was extracted from 1-kg lots of algae by repeated freeze-thawing in 0.1 M potassium phosphate buffer, pH 6.7. The dark blue extracts were clarified by centrifugation at 10,000 × g for 20 min and were brought to 50% saturation with ammonium sulfate. The precipitated C-phycocyanin was sedimented by centrifugation at 10,000 × g for 10 min, dissolved in water, centrifuged at 24,000 × g for 30 min, dialyzed exhaustively against water, lyophilized, and then stored at −15°C.

Chromatography—Analytical high pressure liquid chromatography was performed in a 316-stainless steel column, 0.2 × 20 cm, packed with 5-μm silica gel (E. Merck, Silica gel 60) at 2500 p.s.i. with a flow rate of 0.8 ml min⁻¹. Preparative high pressure liquid chromatography was on a 316-stainless steel column, 1.0 × 25 cm, packed with 5-μm silica gel with a flow rate of 3.0 ml min⁻¹ at 500 p.s.i. The solvent system used was benzene/ethyl acetate/water (80/20/0.13, v/v). Pigments were detected photometrically at 365 nm.

* This research was performed under the auspices of the United States Energy Research and Development Administration.
‡ Present address, Research and Development Department, Colgate-Palmolive Co., Piscataway, New Jersey 08854.
Cleavage—Phycocyanobilin was cleaved from 1-g lots of C-phycocyanin by boiling 150 ml of methanol under reflux with stirring for 15 hours. The dark blue solution was filtered free of protein residue and concentrated to 5 ml. The cleaved discid pigments were converted to their dimethyl esters by boiling under reflux with 7% BF3/methanol for 3 min. Then 10 ml of chloroform and 100 ml of water were added successively to the cooled solution, and the pigment was extracted into the chloroform layer. The chloroform solution was washed with water until neutral and centrifuged at 2000 × g for 5 min. The chloroform layer was concentrated to dryness, dissolved in 5 ml of methanol, and stored at -15°C. The dimethyl esters were purified by preparative high pressure liquid chromatography; the purity of the fractions was assessed by analytical high pressure liquid chromatography.

HCl cleavage of phycocyanobilin was achieved by dissolving 0.5 g of C-phycocyanin in 25 ml of concentrated HCl and holding for 30 min at 25°C. Then 15 ml of ethanol was added to the solution and, after 3 min, 75 ml of water was added. The solution was clarified by centrifugation at 12,000 × g for 5 min, and the pigment was extracted into chloroform. The chloroform solution was washed with water until neutral and concentrated to dryness, and then esterification and purification were carried out as described above.

Phycocyanobilin was enzymatically cleaved from C-phycocyanin with subtilisin BPN' obtained from Sigma Chemical Co., St. Louis, Mo. C-Phycocyanin, 200 mg, was dissolved in 10 ml of 0.1 M sodium phosphate buffer, pH 7.5. The protein was denatured and precipitated by addition of trichloroacetic acid to 5% (w/v). The precipitate was washed twice with 10 ml portions of methanol and twice with 10 ml portions of water with the aid of centrifugation. Subtilisin BPN', 5 mg, was added to the denatured protein dispersed in 20 ml of 0.1 M sodium phosphate buffer, pH 7.5, and the mixture was held for 10 hours at 37°C. The solution was adjusted to pH 4 with acetic acid and extracted twice with 10-ml portions of 1-butanol. The combined 1-butanol extracts were washed four times with 10-ml portions of 1 N acetic acid. The butanol extract was concentrated to dryness by rotary evaporation at 30°C, and the residue was dissolved in methanol. Esterification with 7% BF3/methanol was performed as described above except that the contact time with BF3/methanol was reduced to 1.5 min to minimize adduct formation subsequent to cleavage.

Isomerization—The phycocyanobilin diesters obtained by methanol cleavage of C-phycocyanobilin were boiled under reflux in 10% potassium hydroxide/methanol for 15 min. The alkaline solution was adjusted to pH 4 with acetic acid, and the pigments were extracted into chloroform. The chloroform solution was washed with water until neutral and concentrated to dryness, and then esterification and chromatographic purification were carried out as described above.

Mass Spectra—Mass spectra were obtained by rapid evaporation of samples of pigments dispersed on a Teflon probe. Details of the construction of the sample probe, probe heater, and related apparatus have been described (6). Samples of the order of micrograms of pigment were evaporated completely in time periods of the order of 20’s with heating rates up to 12°C/min.

A quadrupole mass spectrometer (Extranuclear Laboratories) was used for the mass analyses of ions generated from samples of gaseous pigment molecules. A PDP8E computer (Digital Equipment Corp.) was used to operate the mass analyzer controlling the mass range scanned, the scan rate, and the step size within each mass bin scanned.

The computer was also used for data acquisition as well as mass analyzer control. The signal intensity output from a Bendix Channeltron multiplier was recorded as a function of mass and the output of a thermocouple incorporated in the sample probe was also recorded for each repetitive scan of the preselected mass ranges. The minimum step size was 0.11 mass unit and minimum dwell time per point was 2 ms.

Mass spectra were obtained by proton transfer from a primary beam of NH4+ with intensities of approximately 1 × 107 A. Electron impact mass spectra were generated by conventional electron impact ionization techniques at 24 ev and electron currents of the order of 10 to 100 μA. Ionization chamber temperatures were maintained at approximately 125°C and sample evaporation took place at temperatures ranging from 100 to 200°C.

RESULTS AND DISCUSSION

The primary objective of this study is the identification of the several pigments obtained by various chemical cleavage procedures of C-phycocyanin. The structure of phycocyanobilin is shown schematically in Fig. 1a. The products obtained by treatment of the protein with boiling methanol followed by methanol esterification with 7% BF3/methanol were well resolved by high pressure liquid chromatography on silica gel into three components (Fig. 2). Two of these components (Bands I and II) gave virtually identical mass spectra similar to spectra obtained in earlier studies on the phycocyanobilin dimethyl ester (4). The spectrum of the third component was similar to those of Bands I and II except that it gave an intense molecular ion 32 mass units higher than the phycocyanobilin dimethyl ester. Proton transfer and electron impact mass spectra of phycocyanobilin dimethyl ester are presented in Figs. 3 and 4a, respectively, and the electron impact mass spectra of the 32-mass unit adduct of phycocyanobilin dimethyl ester are shown in Fig. 4b. A discussion of these and other spectra shown in Fig. 4 follows.

Several questions emerge in consideration of the three pigments produced in the methanol cleavage reaction. Are these species generated during the cleavage process or subsequent to the cleavage process? Is there indeed a unique pigment-protein complex or are there different structures involved in the generation of the observed cleavage products?

An initial problem was the chemical identification of the
32 mass unit adduct of phycocyanobilin dimethyl ester. Since
the mass-resolving capacity of our quadrupole mass analyzer
could not distinguish between O₂ and CH₃OH at mass 646, it
was not possible to establish the empirical formula of the
adduct by direct measurement. This problem was resolved by
methanol cleavage of C-phycocyanin followed by esterification of
the ethanol-cleaved phycocyanobilin in 7% BF₃/ethanol. After
preparative high pressure liquid chromatography, a derivative
of phycocyanobilin with a molecular weight of 888 was ob-
tained and identified as the ethanol adduct of phycocyanobilin
diethyl ester. Both methanol and ethanol adducts of the
phycocyanobilin diester derivatives were sufficiently stable to
survive evaporation from Teflon surfaces at temperatures
ranging from 100 to 200°.

Experiments designed to determine whether the methanol
adduct is formed during or subsequent to cleavage of pigment
from the protein showed that no adduct was observed when
purified phycocyanobilin dimethyl ester was refluxed with
boiling methanol for time periods ranging from 5 min up to 2
hours. Treatment of phycocyanobilin dimethyl ester with 7%
BF₃/methanol yielded the methanol adduct at a somewhat
slower rate of production than esterification of the phycocyano-
obilin diacid under similar conditions of acid concentration. An
acid catalyst is required for the formation of the adduct. The
possibility that components of the protein itself may be
capable of catalyzing adduct formation exists and indeed must
be seriously considered.

The cleavage of C-phycocyanin with concentrated HCl was
examined. After preparative high pressure liquid chromatogra-
phy, a new pigment was identified by mass spectrometry as the
HCl adduct of phycocyanobilin dimethyl ester. The HCl adduct
survives esterification and the dimethyl ester derivative
gives parent molecule ions at m/e 650 and 652 with the
approximate relative natural abundances of the ³Cl and ³Cl
isotopes. It is also possible to produce an HCl adduct by
treatment of phycocyanobilin dimethyl ester with concen-
trated HCl and re-esterification.

Enzymatic cleavage of phycocyanobilin from C-phycocyanin
was effected with subtilisin RPN' (7). The esterified pigments
from the enzymatic cleavage were compared with methanol-
cleaved pigments by analytical high pressure liquid chroma-
tography. The principal component detected after enzymatic
cleavage was phycocyanobilin dimethyl ester and a trace (less
than 1%) of phycocyanobilin dimethyl ester-methanol adduct.
The enzymatic cleavage yielded other pigment-containing
components, presumably peptides bound to phycocyanobilin,
which did not migrate in the high pressure liquid chromatogra-
phy system described here.

The phycocyanobilin diacids obtained by methanol cleavage
of C-phycocyanin were treated with 10% KOH. The alkaline
treatment isomerized the phycocyanobilin diacids to mesobiliver-
din diacids. Mesobiliverdin and phycocyanobilin are iso-
meric as shown schematically in Fig. 1. The mesobiliverdin
diacids were esterified and chromatographically separated into
three components identified mass spectrometrically as having
molecular weights of 614, 630, and 644. Mesobiliverdin di-
methyl ester has a molecular weight of 614. The 630 molecular
weight compound is assumed to be a water adduct of mesobiliverdin
dimethyl ester that has been dehydrogenated. Simi-
larly the 644 molecular weight compound is considered to be a
dehydrogenated methanol adduct. Mass spectra of the meso-
ibiliverdin adducts differentiate them from phycocyanobilin
derivatives by showing very small relative abundances of ions
at m/e 650, loss of a methyl group from the A ring. Thus, the
mass spectra establish the characteristic A ring unsaturation
of mesobiliverdin in the compounds designated mesobiliverdin
derivatives but provide little or no additional information on
their respective structures. Speculation that follows on the
structures of mesobiliverdin adducts is based on the assump-
tion that mesobiliverdin adducts are derived directly from
phycocyanobilin adducts.

The observation of dehydrogenated mesobiliverdin dimethyl
erster-methanol adduct among the products obtained in the
alkaline isomerization of phycocyanobilin to mesobiliverdin
suggests that the exocyclic double bond in phycocyanobilin is
indeed directly involved in adduct bond formation. If the
adduct molecules were bound to this site, adduct formation
might be more difficult with mesobiliverdin than phycocyanoi-
bin derivatives. If bonding of the adduct molecule occurred at
any site other than the exocyclic double bond of the phycocyano-
obilin tetrapyrrole system, significant differences between
adduct formation with phycocyanobilin and mesobiliverdin
dimethyl ester would not be expected. Experiments in which
purified mesobiliverdin dimethyl ester was refluxed with 7%
BF₃/methanol for 3 min showed no detectable evidence for a
methanol adduct. Under similar circumstances phycocyanobilin
dimethyl ester yielded the methanol adduct in the amount
of at least 10% of the original pigment. This result clearly
indicates that the rate of adduct formation with purified
mesobiliverdin dimethyl ester is at least 1 order of magnitude
less than with the phycocyanobilin derivative. These results
establish the point of attachment of adduct molecules to the
exocyclic olefinic side chain on the A ring of the pigments.

The occurrence of dehydrogenated mesobiliverdin deriv-
atives can be accounted for by the formation of a methanol
adduct of phycocyanobilin during methanol cleavage. The
isomerization of the methanol adduct of phycocyanobilin to
to a mesobiliverdin derivative requires a dehydrogenation reaction:

\[
\begin{align*}
\text{CH}_3 & \quad \text{CHOCH}_3 \\
\text{O} & \\
\text{H} & \\
\text{H} & \\
\text{R} &
\end{align*}
\]

Dehydrogenated mesobiliverdin water and methanol adducts
are not observed to the same extent when purified phycocyano-
obilin dimethyl ester is isomerized to mesobiliverdin. The
isomerization must take place much more rapidly than alka-
line catalysis of adduct formation of phycocyanobilin.

Mass spectroscopy can be used as a technique for molecular
weight determination and to obtain structural information
provided that the systems studied are not significantly per-
turbed in the course of evaporation and ionization processes
used to generate spectra. The tetrapyrrole derivatives, phycocy-
obilin and mesobiliverdin and related compounds, present
problems of volatility which may indeed interfere with mass
spectrometric molecular weight determinations. Cole et al. (4)
and independently Schram and Kroes (2) obtained mass
spectra of phycocyanobilin dimethyl ester which gave a molec-
ular weight of 614. Mass spectrometric studies on phycocyanob-
ilin diacid by Crespi et al. (3) and also by Schram and Kroes
(2) indicated a molecular weight of 588 for the phycocyanobilin
diacid. Schram and Kroes (2) suggested that the molecular
weight of phycocyanobilin was indeed 588 and not 586 as
indicated by work on the dimethyl ester derivatives. They
commented that the diester derivative was incapable of
internal hydrogen bonding to the same extent as the diacid making possible formation of a dehydrogenated verdinoid structure more stable than phycocyanobilin. Since the volatility of the various tetrapyrrole molecular systems is sensitively dependent on hydrogen bonding between molecules, the alternative possibility that phycocyanobilin, m/e 388, was observed in the mass spectrometer because of a hydrogenation reaction must be considered. Hydrogenation reactions of this type have been observed in studies of the mass spectra of complex quinones. The formation of intense M+2 ions by hydrogenation reactions taking place in the course of the evaporation of these relatively nonvolatile molecules has been reported (8-11).

To avoid problems associated with hydrogenation of phycocyanobilin diacids in the course of evaporation in the mass spectrometer, this work was limited to phycocyanobilin diesters. A proton transfer mass spectrum of phycocyanobilin dimethyl ester is presented in Fig. 3. A similar study on mesobiliverdin dimethyl ester gave a protonated parent molecule ion with only traces of decomposition fragments. Protonated phycocyanobilin dimethyl ester is less stable than the corresponding mesobiliverdin compound or it is generated with more internal excitation on proton transfer because of a slightly higher phycocyanobilin ester proton affinity or both. Fragmentations observed with protonated phycocyanobilin dimethyl ester are all accompanied by hydrogen atom rearrangements. For example, the m/e 493 and 126 ions are formed in processes in which the bridge connecting the A or D ring to the rest of the tetrapyrrole system is cleaved. The C=C linkage would normally be considered an unlikely site for bond rupture (cf. Fig. 1a) and one might have expected loss of the methine carbon with the neutral fragment in the formation of a tripyrrole derivative residue instead of the ion at m/e 493. However, the rearrangement of hydrogen suggests the following ionization and dissociation mechanisms:

![Diagram](https://example.com/diagram.png)

given the m/e 493 ion in a process where the conjugated chain of double bonds in the tetrapyrrole system has been broken at the methine bridge between rings C and D. The bond cleaved in this process is not a conjugated double bond but a bond saturated by the proton transfer and hydrogen atom rearrangement process and hence a much weaker and more labile structure. Similar rearrangement mechanisms can account for the formation of ions with masses of 303 and 126, processes which may indeed involve sequential loss of individual rings from the tetrapyrrole system.

The proton transfer spectrum with its minimal fragmentations is of marginal value for structural information particularly with respect to providing information on the location of adduct molecules in tetrapyrrole derivatives. Proton transfer spectra were determined for phycocyanobilin and mesobiliverdin derivatives which showed little more than the evidence for the adduct molecule from the protonated parent molecule ion. The methanol adduct of phycocyanobilin dimethyl ester gave a proton transfer spectrum very similar to that shown in Fig. 3 except for an additional intense ion at m/e 647, the protonated parent molecule ion of the adduct.

Electron impact mass spectra provide more extensive fragmentation which may assist in location of the site of attachment of adduct molecules to tetrapyrrole derivatives. Electron impact mass spectra, in the mass range 285 to 700, of phycocyanobilin and mesobiliverdin dimethyl esters are shown in Fig. 4, a and d. With the exception of the small yields of higher molecular weight species in the mass range 650 to 700 these spectra are very similar to results of Cole et al. (4) and of Schram and Kroes (2). The higher molecular weight ions were not observed in proton transfer spectra on the same materials in the mass spectrometer ion source. The higher molecular weight ions could be produced by a variety of mechanisms, possibly decomposition of ions generated from complex pyrolysis products formed in the evaporation of the phycocyanobilin or mesobiliverdin derivatives. The occurrence of these ion fragments would have been a source of considerable confusion in identifying parent molecule ions if proton transfer or chemical ionization spectra were not available. Their presence indicates the hazards of drawing conclusions on molecular structures based on ions of relatively low intensity in the mass spectrum. These ions are probably artifacts generated in the evaporation of reactive molecules or resulting from impurities formed subsequent to chromatographic purification.

The electron impact mass spectrum of phycocyanobilin and mesobiliverdin derivatives are all characterized by an intense parent molecule ion. The phycocyanobilin and mesobiliverdin dimethyl esters give an intense m/e 614 ion (Fig. 4a and d), the methanol adduct of phycocyanobilin diester an intense m/e 646 ion (Fig. 4b), and the HCl adduct of phycocyanobilin diester an intense m/e 650 ion (Fig. 4c) and isotopic 651 and 652 ions. The phycocyanobilin dimethyl ester is readily identified by the very probable loss of a methyl radical from position 1 of the A ring yielding a relatively intense m/e 599 ion. The m/e 599 ion is also intense in the spectrum of the phycocyanobilin dimethyl ester-methanol adduct. It establishes the presence of a methyl group attached to a saturated carbon atom in one of the rings of the tetrapyrrole systems formed by addition of methanol or HCl to phycocyanobilin dimethyl ester. In contrast, the m/e 599 ion intensities are all very small in all mesobiliverdin spectra as are all fragment ions in the spectra (Fig. 4d), of mesobiliverdin dimethyl ester derivatives, except for the m/e 614 ion of the dehydrogenated methanol adduct (Fig. 4e). The intense ions in this spectrum (Fig. 4e) were generated in ionic ionization and dissociation mechanisms.
Cleavage of Phycocyanobilin

Fig. 4. Partial electron impact mass spectra of: (a) phycocyanobilin dimethyl ester; (b) phycocyanobilin dimethyl ester-methanol adduct; (c) phycocyanobilin dimethyl ester-HCl adduct; (d) mesobiliverdin dimethyl ester; (e) mesobiliverdin dimethyl ester-methanol adduct - $\text{H}_2$, (f) mesobiliverdin dimethyl ester-$\text{H}_2\text{O}$ adduct - $\text{H}_2$. Ion yields decomposition processes or via ionization of mesobiliverdin dimethyl ester produced from the adduct in the course of sample evaporation. A comparison of the relative intensities of the 614 and 644 ions reveals that both are closely parallel in rate of formation as a function of sample heating time (Fig. 5). It is concluded that the m/e 614 ion is formed by decomposition of the parent, m/e 644 ion. Ionic decomposition of parent molecule species to fragment ions occurs in times shorter than the residence times of ions in the mass spectrometer source and transit times through the mass analyzer (time periods of the order of $10^{-5}$ s). Consequently, all products of ionic decomposition must have intensity-time dependences that are identical on the scale of our measurements, a few seconds per spectral scan. Ion species that are generated by neutral decomposition processes on the probe surface may fortuitously have the same intensity-time dependence as products of ionic decomposition. For this to occur, rates of decomposition would have to be followed by more rapid evaporation of the product, and the decomposition rate would have to be approximately the same as the rate of evaporation of the parent molecule ion. These conditions are probably not satisfied in our experiments. The data in Fig. 5 and similar data not shown but taken for the parent and lower molecular weight ions in the phycocyanobilin above m/e 655 were not correlated with the remainder of the spectra and are assumed to be derived from chemically different species produced before or during the evaporation process. Ion yields less than 5% of the parent ion are not presented.

Fig. 5. Relative intensities of parent mesobiliverdin dimethyl ester adduct and m/e 614 fragment ions plotted as a function of sample probe temperature showing the simultaneity of the evolution of neutral molecule precursors of ion species.
considered (12, 13). However, in spite of extensive effort, the sufficient conditions establishing binding of the chromophore to the polypeptide have been inferred from the above observation. Various points of attachment of the pigment in solution. A rigid structure of bound pigment is implied by the observation of fluorescence in the methanol solution.

The evidence for formation of strong bonds with alcohol molecules at the exocyclic olefin linkage as the source of bonding of the adduct molecules comes from unsuccessful attempts to prepare adduct species from purified mesobiliverdin dimethyl esters in BF3/CH3OH. The most conclusive evidence for the identification of the exocyclic olefin linkage as the source of bonding of the adduct species is the observation of the chromophore association reaction spectral data showing a rapid facile gas phase association reaction of protonated phycocyanobilin with neutral gaseous phycocyanobilin in solution. Such reactions may involve bonding of chromophore dimers to a single subunit or dimerization of chromophores already bound to subunits. The latter possibility may occur either within or between subunits contributing to tertiary structure of the system. In any event, models which fail to consider the potential for association of chromophores ignore a potentially important facet of chromophore-protein interactions.

Acknowledgment—We thank N. Alonzo for his cooperation in the chromatographic purification of the pigments.

REFERENCES
Cleavage of Phycocyanobilin

Enzymol. 23, 110–115
Cleavage of phycocyanobilin from C-phycocyanin. Separation and mass spectral identification of the products.
R J Beuhler, R C Pierce, L Friedman and H W Siegelman