Bile Pigment Synthesis in Plants

MECHANISM OF \(^{18}O\) INCORPORATION INTO PHYCOCYANOBILIN IN THE UNICELLULAR RHODOPHYTE, CYANIDIUM CALDARIUM*

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Phycocyanobilin, the bile pigment chromophore of certain phycobiliproteins, is synthesized from \(\delta\)-aminolevulinic acid via the porphyrin pathway and is thought to arise from heme. The origin of the terminal lactam oxygen atoms has been studied by an \(^{18}O\) labeling method developed recently for study of mammalian bile pigment formation.

Cells of the unicellular rhodophyte, Cyanidium caldarium, were incubated in a closed flask in air enriched with \(^{18}O\)O. Phycocyanobilin was isolated from cells and purified as the dimethyl ester (molecular ion, \(M = m/e 614\)) or the dimethyl ester-methanol (covalent) adduct (molecular ion, \(M = m/e 944\)). Quantitative measurement of \(^{18}O\) incorporation into these bile pigments at \(M + 2\) and \(M + 4\) demonstrated phycocyanobilin synthesis by the 2-molecule mechanism. Incorporation of \(^{18}O\) at \(M + 2\) excluded the 1-molecule mechanism in which both lactam oxygen atoms arise from a single oxygen molecule. Incorporation of \(^{18}O\) at \(M + 4\) excluded the hydrolytic mechanism where one lactam oxygen is derived from solvent water and the other from molecular oxygen. \(^{18}O\) incorporation at both \(M + 2\) and \(M + 4\) was inconsistent with the double hydrolytic mechanism which predicts that both lactam oxygen atoms arise from water.

Algal cells incubated for 5 days with \(\delta\)-aminolevulinic acid in medium at pH 1.7 containing 15 atom % \(^{14}C\)O-excreted phycocyanobilin (protein-free). Excreted phycocyanobilin, purified as the dimethyl ester-methanol (covalent) adduct, displayed \(^{18}O\) incorporation at \(M + 2\) and \(M + 4\). Quantitative analysis of \(^{18}O\) incorporation at \(M + 2\) and \(M + 4\) indicated oxygen exchange between solvent water and the lactam oxygen atoms of phycocyanobilin after synthesis and excretion and was inconsistent with the double hydrolytic, the hydrolytic, or the 1-molecule mechanism for synthesis.

The results show that phycocyanobilin in C. caldarium is synthesized by the 2-molecule mechanism in a manner identical to bile pigment formation from heme in mammals, that the mechanism for heme cleavage has been conserved in the evolution of plants and animals, and that the bile pigment chromophores of phycobiliproteins must be produced by an as yet undescribed "algal heme oxygenase."

Phycobiliproteins, of which allophycocyanins, phycocyanins, and phycocyanins are the most important, are accessory photosynthetic pigments found in the red (Rhodophyta), blue-green (Cyanophyta), and cryptomonad (Cryptophyta) algae (1, 2). The light-absorbing chromophore of allophycocyanin and phycocyanin is phycocyanobilin, a linear tetrapyrrole (phycobilin) structurally related to mammalian bile pigment (3–5). The prosthetic group of phycocyanin is phycocerythrin, an open chain tetrapyrrole similar to phycocyanobilin (6–8). Algal bile pigments are covalently linked to apoproteins probably by a thioether bond between the ethylidene side chain of ring I and a cysteinyl residue (9) and possibly by second linkage between the carboxyl group on the propionic acid side chain of ring III and a seryl residue to apoprotein (10) (Fig. 1). Phycobiliproteins can constitute up to 60% of the soluble protein and 25% of the dry weight in cyanophyta species (2) such that a significant portion of the "metabolic effort" in these organisms is directed toward phycobiliprotein biosynthesis.

Algae administered \(\delta\)-aminolevulinic acid synthesized radiolabeled carbon monoxide and phycocyanobilin in a molar ratio of 1:1 (11, 12), and the specific radioactivity of phycocyanobilin was about 7 times greater than that of carbon monoxide. Eight moles of \(\delta\)-aminolevulinic acid are required to make 1 mol of heme, with 4 labeled carbon atoms in the pyrrole rings and 4 labeled carbon atoms in the methene bridges. Thus, the specific radioactivity ratio of phycocyanobilin and carbon monoxide suggests that both compounds are derived from the carbon skeleton of protoporphyrin IX (12).

Although magnesium protoporphyrin IX and iron protoporphyrin (heme) have been discussed as possible intermediates (13), the direct precursor of phycocyanobilin and carbon monoxide has not been established unequivocally. Synthesis of phycocyanobilin from a metal-free porphyrin seems unlikely because porphyrins are not converted to bile pigment in mammalian systems (14, 15) or by chemical oxidation in vitro (16). The conversion of heme to bile pigment in mammals has been extensively investigated (17–19), but the exact sequence of events that occur at the molecular level has not been determined. Overall, heme catalysis in mammals involves oxidative removal of the a-methene bridge carbon atom as carbon monoxide and insertion of 2 new oxygen atoms to produce biliverdin (Fig. 2). Biliverdin is then reduced at the middle methene bridge to form bilirubin and the carbon monoxide formed is exhaled in breath. In mammals, heme is converted to biliverdin by microsomal heme oxygenase (20) and biliverdin is converted to bilirubin by biliverdin reductase (21).

Due to asymmetry of the side chains on the heme pyrrole rings, the methene bridges a, b, γ, and δ are not equivalent and cleavage at these positions can give rise to four isomeric
biliverdins and bilirubins. Bilirubin in bile produced from heme by microsomal heme oxygenase and biliverdin reductase is exclusively the IX α isomer. An explanation for this stereoselectivity has been recently proposed (22). Phycocyanobilin is also the IX α isomer, although in the protein-free chromophore, rings I and IV have ethyliene and ethyl side chains, respectively, rather than vinyl groups (23). This, in conjunction with evidence for the stoichiometric formation of phycocyanobilin and carbon monoxide in algae (12, 24), provide further support for the origin of algal bile pigment from heme.

Tenhunen et al. (25) demonstrated that isotopically labeled molecular oxygen was incorporated into bilirubin synthesized from heme by microsomal heme oxygenase and biliverdin reductase in vitro. Brown and co-workers (26-28) have measured $^{18}$O incorporation into bilirubin in vivo and have demonstrated that the 2 oxygen atoms inserted into precursor molecules of molecular oxygen (2-molecule mechanism). This approach has been instrumental in deciphering the relevance of hypothetical intermediates in heme breakdown postulated for systems. It has also afforded the opportunity to investigate the mechanism of bile pigment synthesis in nonmammalian organisms.

The present investigation describes the incorporation of $^{18}$O-labeled molecular oxygen into phycocyanobilin in the unicellular rhodophyte, *Cyanidium caldarium*, and discusses the relationship between the mechanism of bile pigment synthesis in plants and animals.

**EXPERIMENTAL PROCEDURES**

**Materials**—$^{18}$O$_2$ containing 99.8 atom % $^{18}$O was obtained from Praxair, The British Oxygen Co. Ltd., London SW19 3UF, U. K. $^{16}$O$_2$ and H$_2$O containing 99.0 atom % $^{18}$O was purchased from KOR Isotopes, Cambridge, Mass. Adsorbosil-5 (silica gel) and methanol, 14% BF$_3$ were purchased from Applied Science Laboratories, Inc., State College, Pa. $\Delta$-Aminolevulinic acid hydrochloride was obtained from the Sigma Chemical Co., St. Louis, Mo.

**The Organism** *Cyanidium caldarium* is a unicellular red alga described by Allen (29). When grown heterotrophically in the dark on minimal medium containing 1% glucose (30), the cells divide but do not produce photosynthetic pigments. Dark-grown cells placed in the light in minimal medium minus glucose stop dividing and synthesize chlorophyll $a$, allophycocyanin, and phycocyanin comitantly with manufacturing a single, cup-shaped chloroplast in each algal cell (31). When chloroplast development in the light is completed, pigments are no longer produced and the algal cells remain in a nondividing condition for approximately 7 days. *C. caldarium*, mutant III-D-2, was used in the present work because this strain makes more photosynthetic pigment per cell in the light than does the wild type (32).

**Principle of the $^{18}$O Labeling Method**—The origin of the 2 lactam oxygen atoms incorporated into bile pigment can be determined if the reaction is performed under an atmosphere containing $^{18}$O$_2$ and $^{18}$O$_2$, but not $^{16}$O$_2$. For a molecular ion $M$, peaks at $M + 2$ and $M + 4$ correspond to incorporation of 1 and 2 atoms of $^{18}$O, respectively. Table III shows the predicted incorporation of label into bile pigment for the various mechanisms for three $^{18}$O enrichments in the gas phase. It is clear that the four mechanisms lead to different experimental predictions. In particular, only the 2-molecule mechanism predicts incorporation at both $M + 2$ and $M + 4$.

**Culture Conditions**—The conditions employed for cell growth in the dark and chloroplast development in the light have been described (30).

As a general protocol, dark-grown cells were collected by centrifugation at 30,000 $\times$ g for 10 min, suspended in 3400 ml of minimal medium minus glucose (10$^7$ cells/ml), and placed in a 5400-ml round bottom flask which had been prepared with a stopcock on either side of the neck. The cells were illuminated with fluorescent light (General Electric, power groove, F17PG, CW, approximately 500 foot-candles) with the flask open to air for 18 h. The cells do not make chlorophyll $a$ or phycobiliproteins during this lag period. At the end of the lag period, the flask was closed and 100 to 200 ml of $^{18}$O$_2$ was introduced into the gas phase (2000 ml) above the cell suspension. This was accomplished by creating a slight negative pressure in the flask by aspirating through one stopcock. The negative pressure in the flask was then sufficient to draw in (through the other stopcock) $^{16}$O$_2$ from an inverted, water-filled graduated cylinder into which the isotopically labeled oxygen had been placed. One stopcock was then opened briefly to bring the gas phase in the flask to atmospheric pressure. The closed flask was incubated in the light for 43 to 69 h during which time the cells synthesized chlorophyll $a$ and phycobiliproteins. In a typical experiment, the initial $pO_2$ in the gas phase was 0.28 atm, i.e. 360 ml ($^{16}$O$_2$) + 200 ml ($^{18}$O$_2$)/2000 ml (volume of gas phase). Gas samples (10 to 30 ml) were removed from the gas phase at intervals during the experiments. This produced a slight negative pressure in the flask which prevented cell sampling for quantitative analyses of pigment synthesis.

**Phycobiliprotein Isolation**—Algal cells were collected by centrifugation, resuspended in 0.1 m phosphate buffer (KH$_2$PO$_4$/K$_2$HPO$_4$),
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pH 7.0, and disrupted by sonic vibration with a Branson sonic oscillator (model W185) as described previously (39). The temperature of the cell suspension (1 ml of packed cells/10 ml of buffer) was kept below 20°C by cooling in an ice bath. After sonic disruption of the cells, the sample was centrifuged at 36,000 × g for 30 min to remove particulate material and the resulting supernatant was saturated with ammonium sulfate. The precipitate (proteins including allophycocyanin and phycocyanin in addition to other materials insoluble in ammonium sulfate) was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water at 4°C. After dialysis, the samples were centrifuged at 100,000 × g for 1 h at 4°C to remove traces of chlorophyll a, and frozen at −20°C until used.

**Absorption of Phycocyanobilin**—Trichloroacetic acid was added to dialyzed and ultracentrifuged samples to make the final concentration 1%, and the samples were incubated at room temperature for 3 h with constant stirring. The precipitated material including allophycocyanin and phycocyanin was collected by centrifugation, washed three times with 20 ml of distilled water, and three times with 20 ml of absolute methanol. The dried sample was suspended in 500 ml of absolute methanol and heated at 60°C for 16 h. The methanol was cooled, filtered through Whatman No. 1 filter paper to remove denatured protein and other debris, and the filtrate containing phycocyanobilin-free acid was evaporated to dryness under reduced pressure at 50°C.

**Esterification**—The phycocyanobilin residue was dissolved in 5 ml of absolute methanol, an equal volume of methanol, 14% BF₃ was added and the solution was heated at 60°C for 2 to 3 min. The solution was cooled, an equal volume of chloroform and 10 volumes of distilled water were added, and the resulting chloroform phase was washed with 10 ml of distilled water three times. The chloroform solution containing esterified bile pigments was filtered through chloroform-moistened filter paper and evaporated to dryness under a stream of nitrogen, and the residue was stored at −20°C until used.

**Thin Layer Chromatography**—The residue from the esterification procedure was dissolved in chloroform, applied to silica gel (Adsorbosil-5, 500 µ thick) plates (20 × 20 cm) which were developed in carbon tetrachloride:methyl acetate (2:1, v/v). Two major bands were observed. One of these was phycocyanobilin dimethyl ester (RF = 0.95) and the other was phycocyanobilin dimethyl ester-methanol (covalent) adduct (RF = 0.44). The latter compound has been described in detail by Beuhler et al. (34).

**Mass Spectrometry**—Mass spectra of bile pigments were determined on an AEI M.S. 9 mass spectrometer. Mass spectra of gas samples were recorded on an AEI M.S. 10 mass spectrometer. From these gas samples, it was possible to determine the partial pressure of both 16O and 18O, and, hence, the total oxygen pressure (pO₂) and also the percentage of 18O (i.e. 18O/16O + 18O). Incorporation of O₁₈O from Isotopically Labeled Water into Phycocyanobilin—An attempt was made to determine whether oxygen from H₂¹₈O was incorporated into the lactam oxygen atoms on rings I and IV of phycocyanobilin. The cost of H₂¹₈O prevented direct examination of phycocyanobilin synthesis (covalently attached to apoprotein) by techniques employed in experiments with ¹⁸O₂. Therefore, advantage was taken of the novel process in which C. caldarium cells incubated with d-aminolevulinic acid excrete protein-phycocyanobilin (free acid) into the suspending medium (35, 36).

**Results**

**Chromophore Cleavage and Esterification**—The covalent linkage between phycocyanobilin and apoprotein of phycocy- anin and allophycocyanin is cleaved in boiling methanol releasing the chromophore-free acid. Esterification of phycocyanobilin in methanol, 7% BF₃ yielded the dimethyl ester and the dimethyl ester-methanol (covalent) adduct (Fig. 3). When more than 500 µg of phycocyanobilin was esterified, the ratio of dimethyl ester to dimethyl ester-methanol (covalent) adduct was usually about 1:1. The use of less than 200 µg of phycocyanobilin resulted in a ratio of dimethyl ester to dimethyl ester-methanol (covalent) adduct of 1:10. The reason for this result is not known. Chromic acid degradation of phycocyanobilin by modification (37) of the procedure of Rudiger and O’Carra (10) yielded ring I as 1-methyl-2-ethylidene succinimide. Chromic acid degradation of phycocyanobilin dimethyl ester-methanol (covalent) adduct yielded ring I as an imide tentatively identified (37) as methyl-a-methox- yethylmaleimide. We are reasonably certain, therefore, that the methanol adduct was produced during esterification and not during chromophore cleavage in boiling methanol.

Absorption spectra and Rₚ values of phycocyanobilin di- methyl ester and phycocyanobilin dimethyl ester-methanol (covalent) adduct are given in Table I. The electron impact mass spectrum of each bile pigment diester in the region from 3413 to 3416 Da is shown in Fig. 4.

**Table I**

<table>
<thead>
<tr>
<th>Bile pigment</th>
<th>Chlororm</th>
<th>Methanol, 5% HCl</th>
<th>Rₚ value*</th>
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<tr>
<td></td>
<td>λ₁</td>
<td>λ₂/λ₃</td>
<td>λ₁</td>
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<td>Phycocyanobilin dimethyl ester</td>
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<td>Mesobiliverdin dimethyl ester</td>
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* Rₚ values of bile pigment diesters were determined on Adsorbosil-5 silica gel plates developed with carbon tetrachloride:methyl acetate (2:1, v/v).
m/e 595 to m/e 650 is indicated in Fig. 4. The mass spectrum of phycocyanobilin dimethyl ester had major peaks at m/e 614, 599, 493, and 302, and minor peaks at m/e 583, 555, and 541, in excellent agreement with published values (34).

The molecular ion, M, of phycocyanobilin dimethyl ester-methanol (covalent) adduct occurred at m/e 644. Accurate mass measurements indicated a molecular weight of 644.3298 and elementary composition of C₉₀H₅₀N₂O₇ (exact mass of 644.3210; 2.8 ppm error). Beuhler et al. (34) reported that the molecular ion of the dimethyl ester-methanol (covalent) adduct occurred at m/e 646 with a relatively minor peak at m/e 644. Their spectra were obtained at temperatures between 100 and 200°C under special conditions of sample evaporation from a Teflon probe. We were unable to obtain mass spectra of the phycocyanobilin dimethyl ester-methanol (covalent) adduct prepared from cells in which phycobiliprotein synthesis occurred in air (¹⁸O₂) and air enriched with ¹⁸O₂. A, phycocyanobilin dimethyl ester, air. Mass spectra were recorded at 240°C. B, phycocyanobilin dimethyl ester, air enriched with ¹⁸O₂. The initial isotope level was 34.6% ¹⁸O and the final isotope level was 15.7% ¹⁸O. The mass spectra were recorded at 240°C and were used to obtain the data for O incorporation in Experiment 2 in Table III. C, phycocyanobilin dimethyl ester-methanol (covalent) adduct, air. The peaks at m/e 644 and m/e 645 correspond closely with values calculated for the naturally abundant isotope peaks relative to m/e 644. This demonstrates that there was no contribution at m/e 646 from species with molecular weight higher than 644. The mass spectra were recorded at 190°C. D, phycocyanobilin dimethyl ester-methanol (covalent) adduct, air enriched with ¹⁸O₂. The initial isotope level was 35.0% ¹⁸O and the final isotope level was 15.6% ¹⁸O. The mass spectra were recorded at 200°C and were used to obtain the data showing ¹⁸O incorporation into algal bile pigment in Experiment 3, Table III.

![Fig. 4. Partial mass spectra of phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester-methanol (covalent) adduct prepared from cells in which phycobiliprotein synthesis occurred in air (¹⁸O₂) and air enriched with ¹⁸O₂. A, phycocyanobilin dimethyl ester, air. Mass spectra were recorded at 240°C. B, phycocyanobilin dimethyl ester, air enriched with ¹⁸O₂. The initial isotope level was 34.6% ¹⁸O and the final isotope level was 15.7% ¹⁸O. The mass spectra were recorded at 240°C and were used to obtain the data for O incorporation in Experiment 2 in Table III. C, phycocyanobilin dimethyl ester-methanol (covalent) adduct, air. The peaks at m/e 644 and m/e 645 correspond closely with values calculated for the naturally abundant isotope peaks relative to m/e 644. This demonstrates that there was no contribution at m/e 646 from species with molecular weight higher than 644. The mass spectra were recorded at 190°C. D, phycocyanobilin dimethyl ester-methanol (covalent) adduct, air enriched with ¹⁸O₂. The initial isotope level was 35.0% ¹⁸O and the final isotope level was 15.6% ¹⁸O. The mass spectra were recorded at 200°C and were used to obtain the data showing ¹⁸O incorporation into algal bile pigment in Experiment 3, Table III.](http://www.jbc.org/)

![Fig. 5. Variation in the pO₂ (A) and percentage of ¹⁸O (B) in the gas phase above cells in the closed flask during phycobiliprotein synthesis. The data for ¹⁸O incorporation into algal bile pigment (in this experiment) correspond to those in Experiment 2, Table III.](http://www.jbc.org/)
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at temperatures below 190°C. Bile pigments can be perturbed during sample evaporation and ionization in the mass spectrometer. We conclude that the peak observed at m/e 644 was due to phycocyanobilin dimethyl ester-methanol (covalent) adduct minus H₂.

Since both phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester-methanol (covalent) adduct are derivatives of phycocyanobilin synthesized in C. caldarium cells, mass spectral measurements on both diesters have been used in ¹⁸O labeling experiments in the present work.

Percentage of ¹⁸O in the Gas Phase above Algal Cells—The pO₂ and percentage of ¹⁸O in the gas phase were measured by mass spectrometry at intervals throughout each experiment. Mass spectra of gas samples displayed peaks at m/e 32 and m/e 36 corresponding to ¹⁶O, ¹⁸O, and ¹⁸O₂, respectively.

Subtracting the small contributions yields the residuals shown in Column 3. As expected, the residual at m/e 645 is relatively very small and may represent the proportionate error in the method (and thus be effectively zero) or it may represent a very small, but real, impurity in the sample. Assuming the latter, there will be naturally abundant isotopic peaks associated with this small residual and, assuming the same ratios as shown in Column 2, these are entered in Column 4. At no time did a peak appear at m/e 34 which might signal the formation of ¹⁸O₂ molecules. Formation of mixed (hybrid) molecules would render ¹⁸O labeling patterns in phycocyanobilin ambiguous and uninterpretable. The results obtained from one such experiment are shown in Fig. 5. The pO₂ decreased from 0.27 to 0.15 atm in the first 24 h of incubation in the closed flask. Subsequently, the pO₂ increased to nearly the initial level of 0.27 atm but the partial pressure of ¹⁸O₂, which had fallen to 0.04 atm, did not change significantly.

¹⁸O Incorporation into Phycocyanobilin—¹⁸O was incorporated into phycocyanobilin in C. caldarium cells incubated in air enriched with ¹⁸O₂ (Fig. 4). The mass spectrum of phycocyanobilin dimethyl ester showed ¹⁸O incorporation at m/e 616 (M + 2) and m/e 618 (M + 4). Similarly, the mass spectra of phycocyanobilin dimethyl ester-methanol adduct minus H₂ displayed peaks at m/e 644, 645, 646, and 648.

Subtracting the small contributions yields the residuals shown in Column 8. Subtracting Column 8 from Column 7 yields the true value of the peak at m/e 648 corresponding to ¹⁸O incorporation (Column 9). Column 10 summarizes the true ¹⁸O incorporation at M + 2 and M + 4 for the double hydrolytic, hydrolytic, and 1-molecule mechanisms.

<table>
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<tr>
<th>Experiment</th>
<th>Compound</th>
<th>m/e</th>
<th>Final ¹⁸O₂ Concentration</th>
<th>Double Hydrolytic Mechanism</th>
<th>Hydrolytic Mechanism</th>
<th>1-Molecule Mechanism</th>
<th>2-Molecule Mechanism</th>
<th>Observed ¹⁸O₂</th>
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<td></td>
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<td>86.7</td>
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<td></td>
<td>methanol (covalent) adduct</td>
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<td>22.6</td>
<td>16.5</td>
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<td>0</td>
<td>13.0</td>
<td>1.7</td>
<td>1.0</td>
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Subtracting the small contributions yields the residuals shown in Column 3. As expected, the residual at m/e 645 is relatively very small and may represent the proportionate error in the method (and thus be effectively zero) or it may represent a very small, but real, impurity in the sample. Assuming the latter, there will be naturally abundant isotopic peaks associated with this small residual and, assuming the same ratios as shown in Column 2, these are entered in Column 4. At no time did a peak appear at m/e 34 which might signal the formation of ¹⁸O₂ molecules. Formation of mixed (hybrid) molecules would render ¹⁸O labeling patterns in phycocyanobilin ambiguous and uninterpretable. The results obtained from one such experiment are shown in Fig. 5. The pO₂ decreased from 0.27 to 0.15 atm in the first 24 h of incubation in the closed flask. Subsequently, the pO₂ increased to nearly the initial level of 0.27 atm but the partial pressure of ¹⁸O₂, which had fallen to 0.04 atm, did not change significantly.

¹⁸O Incorporation into Phycocyanobilin—¹⁸O was incorporated into phycocyanobilin in C. caldarium cells incubated in air enriched with ¹⁸O₂ (Fig. 4). The mass spectrum of phycocyanobilin dimethyl ester showed ¹⁸O incorporation at m/e 616 (M + 2) and m/e 618 (M + 4). Similarly, the mass spectra of phycocyanobilin dimethyl ester-methanol adduct minus H₂ displayed peaks at m/e 644, 645, 646, and 648.

Subtracting the small contributions yields the residuals shown in Column 8. Subtracting Column 8 from Column 7 yields the true value of the peak at m/e 648 corresponding to ¹⁸O incorporation (Column 9). Column 10 summarizes the true ¹⁸O incorporations and in Column 11, they are shown normalized in terms of mol percent of phycocyanobilin dimethyl ester-methanol (covalent) adduct at the three mass numbers. These are the parameters that are used in subsequent analysis.
Mechanism of Phycocyanobilin Synthesis

TABLE IV

<table>
<thead>
<tr>
<th>Bile pigment</th>
<th>m/e</th>
<th>Peak</th>
<th>Size of peak (relative)</th>
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<td>Phycocyanobilin dimethyl ester-methanol</td>
<td>646</td>
<td>M</td>
<td>81.7 99.2</td>
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<tr>
<td>Phycocyanobilin dimethyl ester-methanol (covalent) adduct</td>
<td>646</td>
<td>M + 2</td>
<td>17.2 0.8</td>
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<tr>
<td>Phycocyanobilin dimethyl ester-methanol (covalent) adduct</td>
<td>646</td>
<td>M + 4</td>
<td>1.1 0.0</td>
</tr>
</tbody>
</table>

The spectrum of the dimethyl ester-methanol (covalent) adduct indicated $^{18}$O incorporation at m/e 646 (M + 2) and m/e 648 (M + 4). Relative peak heights were determined as the mean of 12 to 15 scans in each case. After correction for the naturally abundant isotopes $^{12}$C and $^{14}$N (28), residual peaks at M + 2 and M + 4 correspond to the experimental incorporation of $^{18}$O. The method is illustrated for one experiment in Table II.

Table III shows the incorporation at M + 2 and M + 4 in three separate experiments, compared to the predicted labeling patterns for the various mechanisms outlined above. Predicted (calculated) values were based on the final $pO_2$ of 18.1802 in the gas phase in each experiment. Since phycocyanin synthesis probably occurred during a falling $^{18}$O/$^{16}$O ratio, immediate quantitative interpretation of the data is difficult. However, qualitatively, it is clear that incorporation occurred at both M + 2 and at M + 4 in each experiment. The double hydrolytic and hydrolytic mechanisms are excluded by the observation of incorporation at M + 4. The 1-molecule mechanism is incompatible with incorporation at M + 2 which, in each case, was substantial. Only the 2-molecule mechanism predicts incorporation at both M + 2 and M + 4. Consequently, it is clear that, on this basis, phycocyanobilin synthesis occurs by the 2-molecule mechanism.

Incorporation of $^{18}$O From H$_2$18O into Phycocyanobilin—C. caldarium cells incubated in the dark with H$_2$18O (15 atom %, see “Experimental Procedures”) yielded a relatively small quantity of phycocyanobilin. Upon esterification and purification, insufficient phycocyanobilin dimethyl ester was obtained for mass spectrometry and measurements were restricted to phycocyanobilin dimethyl ester-methanol (covalent) adduct.

Significant $^{18}$O incorporation was observed in the dimethyl ester-methanol (covalent) adduct at m/e 646 (M + 2) and m/e 648 (M + 4) after correction for natural isotopes (Table IV). Peaks were not observed at m/e 650 (M + 6) or at m/e 652 (M + 8). Quantitative analyses of these data demonstrate that $^{18}$O in excreted phycocyanobilin can be ascribed to oxygen exchange with solvent water (see “Discussion”).

DISCUSSION

The biochemical events which occur in C. caldarium cells in the lag period prior to the time when dark-grown cells begin making photosynthetic pigment have not been hitherto determined. It has been assumed that reserve carbohydrate (starch) is mobilized and respired for energy-requiring processes in chloroplast development in the lag period and in the initial interval of pigment synthesis. Respiration would become unnecessary at the point in chloroplast development when the cells become photosynthetically competent.

That this occurs in C. caldarium has been confirmed by measurements of the $pO_2$ and percentage of $^{18}$O in the gas phase above the algal cell suspensions incubated in the closed flask in the light (Fig. 5). The initial decrease in the $pO_2$ demonstrates that the cells respire prior to the time that significant quantities of chlorophyll and phycobiliproteins are produced. During this time, the percentage of $^{18}$O changed little as might be expected, since both isotopes would be utilized approximately equally for respiration. However, after about 24 h in the closed flask, the $pO_2$ value passed through a minimum and began to increase again, suggesting the onset of photosynthesis and the production of oxygen (i.e. $^{16}$O$_2$ from water). Correspondingly, the $^{18}$O/$^{16}$O ratio decreased sharply at this stage. Fig. 5 also shows that after about 24 h, the partial pressure of $^{18}$O$_2$ remained relatively constant, demonstrating the cessation of respiration. Finally, the $pO_2$ value leveled out at approximately its initial value, presumably because the closed atmosphere became depleted of carbon dioxide. Had respiration continued, there would have been a gradual decrease in the partial pressure of $^{18}$O$_2$.

Initially, we were surprised to observe $^{18}$O incorporation into phycocyanobilin synthesized from $\delta$-aminolevulinic acid and excreted from cells incubated with H$_2$18O. The absence of $^{18}$O in excreted phycocyanobilin would have confirmed the experiments performed under 18.1802, which were inconsistent with the double hydrolytic, the hydrolytic, or the 1-molecule mechanism. This expectation was perhaps overly optimistic in view of the fact that the excreted phycocyanobilin was exposed to H$_2$18O at 37°C for 5 days at pH 1.7.

The observation of $^{18}$O incorporation from H$_2$18O has important consequences with respect to experiments demonstrating $^{16}$O incorporation into phycocyanobilin bound to apoprotein in vivo. There are three explanations for the incorporation of isotopically labeled oxygen from H$_2$18O into the excreted phycocyanobilin. First, incorporation of $^{18}$O from solvent water could indicate the double hydrolytic mechanism or the hydrolytic mechanism for phycocyanobilin synthesis. However, incorporation of $^{18}$O (from water) at M + 4 is not predicted by the hydrolytic mechanism. This, together with incorporation of $^{16}$O at M + 2 is compelling evidence against either the double hydrolytic mechanism or the hydrolytic mechanism.

Second, oxygen exchange could have been restricted to oxygen atoms in the carboxyl groups on rings II and III. Since peaks at M + 6 and M + 8 were not observed, there could be a maximum of one $^{18}$O per carboxyl group on average. Had exchange occurred between H$_2$18O and the —CO$_2$ groups, one would expect that each oxygen atom in the carboxyl group would have an equal chance of being exchanged. One oxygen atom in each carboxyl group is eliminated after esterification which adds a CH$_3$O— group. This would mean that the maximum $^{18}$O level in phycocyanobilin (assuming complete exchange) would be one-half of that of labeled water. The observed $^{18}$O incorporation from H$_2$18O into phycocyanobilin was two-thirds of that of labeled water (Table IV). That is, $^{18}$O in phycocyanobilin was $^{18}$O(1/2 + 2 x 1.1) = 9.5 atom %, whereas the $^{18}$O level in water was 15 atom %. This would seem to exclude the possibility that $^{18}$O in the excreted phycocyanobilin can be ascribed to oxygen exchange between solvent water and oxygen atoms in the carboxyl groups on rings II and III.

Third, oxygen exchange may have occurred exclusively between the lactam oxygen atoms on rings I and IV and solvent water (H$_2$16O). If correct, the reaction would predict a small peak at M + 4 because there is a small chance that both lactam oxygen atoms would be exchanged. Since the observed exchange was two-thirds complete after 5 days (9.7 atom % in phycocyanobilin/15 atom % in water), the exchange reaction is relatively slow. Furthermore, for 9.7 atom % $^{18}$O in phycocyanobilin, the predicted incorporation at M, M + 2, and M + 4 (dimethyl ester-methanol (covalent) adduct) would be:
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m/e 644, (0.903 × 0.903 × 100) = 81.5%; m/e 646, (2 × 0.903 × 0.907 × 100) = 9.3%. These calculated values are in excellent agreement with the experimental results (Table IV). They appear to confirm that the exchange reaction is quite slow and that it occurs subsequent to phycocyanobilin synthesis.

Exchange with solvent water might be expected to lead to an apparent decrease in the observed incorporation in vivo of 18O into phycocyanobilin attached to apoprotein in the C. caldarium chloroplast. This exchange would be small on the basis of the experiment done with H218O because the incubation times in experiments done under 18O2 were shorter (43 to 69 h versus 5 days, see Tables IV and V) and the effect of chromophore binding to apoprotein would probably reduce exchange further although this would be difficult to determine experimentally.

Table V shows values for total incorporation of 18O into phycocyanobilin for Experiments 1 to 3 (Table III), which reveal that such exchange does occur. In Experiments 1 and 2 (Table III) with isotopically labeled molecular oxygen, the maximum labeling in phycocyanobilin dimethyl ester or phycocyanobilin dimethyl ester-methanol (covalent) adduct was less than the minimum 18O level in the gas phase at the end of the experiment. For example, in Experiment 1, the initial 18O level in the gas phase was 10.4%. Since the 18O in phycocyanobilin dimethyl ester was 9.3%, this would suggest a maximum exchange of 10.4% − 9.3%/10.4 × 100 = 10.5% had occurred. The minimum exchange in Experiment 2 (Table III) would be 28.5%. The "true" exchange would be greater because some of the phycocyanobilin was synthesized when the 18O level in the gas phase above the cell suspension was greater than 10.4% (e.g. Fig. 5). Nevertheless, the order of magnitude of the calculated exchange for the incubation times involved is compatible with the experiments carried out with H218O.

The question arises whether the exchange reaction causes doubt on the distinction between the 2-molecule mechanism and the 1-molecule mechanism since exchange with solvent water could produce a peak at M + 2 if initially there was none.

Consider the case of phycocyanobilin synthesis by a 1-molecule mechanism followed by exchange, using Experiment 1 (Table V) as an example. After initial pigment synthesis, the 1-molecule mechanism would predict 10.4% incorporation at M + 4 and zero at M + 2. Exchange would involve a progressive decrease in the 2-molecule M + 4 species, eventually to zero when exchange is complete. For M + 2, the apparent incorporation would initially rise from zero, would go through a maximum, and then decrease to zero when exchange was complete. The maximum value for M + 2 can be shown algebraically.

Let m be the concentration of the M + 4 species at time t, and n be the concentration of the M + 2 species at time t, then, (dm/dt) = −km where k is the rate constant for exchange, and (dn/dt) = km − kn where k is the rate constant for exchange. The solution to these differential equations is: m = m0 e−kt where m0 is the concentration of m at time zero; n = kn0e−kt where n0 is the concentration of n at time zero. Differentiating with respect to time, it can be shown that n is maximum when t = 1/k, i.e. m = n = m0/e.

Thus, for 10.4% incorporation at M + 4, the maximum value at M + 2 for a 1-molecule mechanism followed by exchange is 10.4/2.72 = 3.8%. The observed value at M + 2 was 16.5% which is well above the maximum calculated value. Even in the extreme case where the 18O level in the gas phase remained at its initial value of 26.3%, the maximum calculated value of M + 2 would be 26.3/2.72 = 9.7%, again well below the observed value. It is, therefore, impossible to correlate the observed incorporation values in these experiments with one 1-molecule mechanism, even allowing for exchange.

In the case of the 2-molecule mechanism, initial synthesis in Experiment 1 (Table V) at 10.4% 18O in the gas phase would predict M + 2 = 18.6% and M + 4 = 11.1%. If one assumes a progressive exchange after initial synthesis of phycocyanobilin, there would be a gradual decrease in both M + 2 and M + 4. Since the initial incorporation at M + 4 is small, exchange from this makes little contribution to M + 2, even though every exchange event at M + 4 produces M + 2, whereas only one-half of the exchange events at M + 2 results in loss of M + 2. Since the observed incorporation at M + 2 was 16.5% and the predicted value for M + 2 (assuming 10.4% 18O in the gas phase) was 18.7%, this exchange is 18.7 − 16.5/18.7 × 100 = 12%. This exchange is reasonable on the basis of the exchange observed in the experiment with H218O. Similar analyses of the data for Experiments 2 and 3 (Table III) show that there is no doubt that phycocyanobilin is synthesized by the 2-molecule mechanism but that there is also some exchange occurring.

The confirmation that algal bile pigment synthesis proceeds via the same mechanism with respect to oxygen as does mammalian bile pigment synthesis may be regarded as further evidence for the involvement of heme in the pathway of phycobiliprotein biosynthesis. This suggests the conservation of the mechanism of heme-cleaving systems during evolution and represents circumstantial evidence for the existence of an "algal heme oxygenase," although such an enzyme has not yet been detected.

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