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 \( ^{18} \text{O} \) labeling method developed recently for study of mammalian bile pigment formation.

Cells of the unicellular rhodophyte, \textit{Cyanidium caldarium}, were incubated in a closed flask in air enriched with \( ^{18} \text{O} \)\(_2\). Phycocyanobilin was isolated from cells and purified as the dimethyl ester (molecular ion, \( M = m/e \) 644). Quantitative measurement of \( ^{18} \text{O} \) incorporation into these bile pigments at \( M + 2 \) and \( M + 4 \) demonstrated phycocyanobilin synthesis by the 2-molecule mechanism. Incorporation of \( ^{18} \text{O} \) at \( M + 2 \) and \( M + 4 \) excluded the 1-molecule mechanism in which both lactam oxygen atoms arise from a single oxygen molecule. Incorporation of \( ^{18} \text{O} \) at \( M + 4 \) excluded the hydrolytic mechanism where one lactam oxygen is derived from solvent water and the other from molecular oxygen. \( ^{18} \text{O} \) 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 \% \( H_2^{18} \text{O} \)-excreted phycocyanobilin (protein-free). Excreted phycocyanobilin, purified as the dimethyl ester-methanol (covalent) adduct, displayed \( ^{18} \text{O} \) 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 \textit{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 phycocerythrins 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 phycocrytbilin, 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 carbonyl 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.

Phycocyanobilin and carbon monoxide 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 \textit{vitro} (16).

Due to asymmetry of the side chains on the heme pyrrole rings, the methene bridges \( \alpha, \beta, \gamma \), and \( \delta \) are not equivalent and cleavage at these positions can give rise to four isomeric...
biliarverdins and bilirubins. Bilirubin in bile produced from heme by microsomal heme oxygenase and biliverdin reductase is exclusively the IX a isomer. An explanation for this stereoselectivity has been recently proposed (22). Phycocyanobilin is also the IX a isomer, although in the protein-free chromophore, rings I and IV have ethylidine 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 18,18O 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 from chemical oxidation of heme in vitro to intermediates produced during physiologic heme turnover in mammalian 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 18O-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—**18,18O2 containing 99.8 atom % 18O was obtained from Praxair, The British Oxygen Co. Ltd., London SW19 3UF, U. K. 16,16O2 and H16O containing 99.0 atom % 18O was purchased from KOR Isotopes, Cambridge, Mass. Adsorbosil-5 (silica gel) and methanol, 14% BF3 were purchased from Applied Science Laboratories, Inc., State College, Pa. 8-Aminolevulinic acid hydrochloride was obtained from the Sigma Chemical Co., St. Louis, Mo.

*The Organism*—Cyanidium caldarium is a unicellular red alga

![Fig. 1. Proposed covalent attachment of phycocyanobilin to apoprotein (10).](http://www.jbc.org/)

**Principle of the 18O 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 18,18O2 and 16,16O2, but not 18,16O2 (26-28). Four possible mechanisms may be defined: (a) the double hydrolytic mechanism (both oxygen atoms derived from solvent water); (b) the hydrolytic mechanism (1 oxygen atom from solvent water and the other from molecular oxygen); (c) the 1-molecule mechanism (both oxygen atoms from a single oxygen molecule); (d) the 2-molecule mechanism (both oxygen atoms from molecular oxygen but from different oxygen molecules). These mechanisms may be distinguished experimentally by mass spectrometry of the bile pigment formed under a mixture of 18,18O2 and 16,16O2. For a molecular ion M, peaks at M + 2 and M + 4 correspond to incorporation of 1 atom from solvent water and 2 atoms from molecular oxygen (12, 24). Table III shows the predicted incorporation of label into bile pigment for the various mechanisms for three 18O 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 x g for 10 min, suspended in 3400 ml of minimal medium minus glucose (109 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 18,18O2 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) 16,16O2 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 p02 in the gas phase was 0.28 atm, i.e. 360 ml (16O) + 200 ml (18O)/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 (KH2PO4/K2HPO4),...
Mechanism of Phycocyanobilin Synthesis

pH 7.0, and disrupted by sonic vibration with a Branson sonic oscillator (model W185) as described previously (33). 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. 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. The residue was stored at −20°C by cooling in an ice bath. After sonic disruption of the cells, the sample was centrifuged at 36,000 × g 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). Mass spectra of bile pigments were determined on an AEI M.S. 10 mass spectrometer. From these gas samples, it was possible to determine the partial pressure of both 18O₂ and 13CO₂ and, hence, the total oxygen pressure (pO₂) and also the percentage of 18O (i.e. 18O/H₂O + 16O).

Incorporation of 18O 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 δ-aminolevulinic acid excrete protein-free phycocyanobilin (free acid) into the suspending medium (35, 36).

C. caldarium cells were incubated in the light as described above for 5 days. The pigmented cells (1.5 ml packed cell volume) were suspended in 8.5 ml of minimal medium (pH 7.0) containing 15% Hz¹⁸O (1.5 ml of 99% H₂¹⁸O), 1.5 × 10⁻² M glucose, and 5 × 10⁻⁵ M δ-aminolevulinic acid. The cell suspension was incubated for 5 days in the dark at 37°C on a rotary shaker. After incubation, the cells were collected by centrifugation and the supernatant was extracted three times with 2 ml of chloroform. The chloroform extract containing phycocyanobilin free acid was washed three times with 10 ml of distilled water, filtered through chloroform-moistened filter paper, and evaporated to dryness under a stream of nitrogen. Phycocyanobilin in the residue was esterified and purified by thin layer chromatography as described above.

RESULTS

Chromophore Cleavage and Esterification—The covalent linkage between phycocyanobilin and apoprotein of phycocyanin 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-methoxymethylmaleimide. We are reasonably certain, therefore, that the methanol adduct was produced during esterification and not during chromophore cleavage in boiling methanol.

Absorption spectra and Rf values of phycocyanobilin dimethyl 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 199 to 341 mass units is shown in Fig. 4.
Mechanism of Phycocyanobilin Synthesis

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 C_{20}H_{24}N_{2}O_{2} (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 in the temperature range above 200°C because the fragmentation patterns in the mass spectra were different at these higher temperatures.

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 (\(^{16}O_2\)) and air enriched with \(^{18}O_2\). A, phycocyanobilin dimethyl ester, air. Mass spectra were recorded at 240°C. B, phycocyanobilin dimethyl ester, air enriched with \(^{18}O_2\). The initial isotope level was 37.4% \(^{18}O\) and the final isotope level was 15.5% \(^{18}O\). The mass spectra were recorded at 240°C and were used to obtain the data for \(^{18}O\) incorporation in Experiment 2 in Table III. C, phycocyanobilin dimethyl ester-methanol (covalent) adduct, air. The peaks at m/e 645 and m/e 646 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 \(^{18}O_2\). The initial isotope level was 35.0% \(^{18}O\) and the final isotope level was 15.5% \(^{18}O\). The mass spectra were recorded at 200°C and were used to obtain the data showing \(^{18}O\) incorporation into algal bile pigment in Experiment 3, Table III.

FIG. 5. Variation in the \(pO_2\) (A) and percentage of \(^{16}O\) (B) in the gas phase above cells in the closed flask during phycobiliprotein synthesis. The data for \(^{18}O\) incorporation into algal bile pigment (in this experiment) correspond to those in Experiment 2, Table III. Synthesis of photosynthetic pigments was first visibly detectable about 10 h after \(^{16,18}O_2\) was introduced and the flask was closed, and appeared to continue throughout incubation for 48 h.
Mechanism of Phycocyanobilin Synthesis

Phycocyanobilin was cleaved from phycobiliproteins in boiling methanol, purified as the dimethyl ester and dimethyl ester-methanol (covalent) adduct, and analyzed in the mass spectrometer. 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 ¹⁶O₂ and ¹⁸O₂, respectively.

Subtracting the small contributions yields the residuals shown in Column 7. As with the residual at m/e 647 in Column 5, the very small residual at m/e 648 corresponding to ¹⁰O incorporation at m/e 616 (M + 2) and m/e 618 (M + 4). Similarly, the mass spectrum of phycocyanobilin dimethyl ester showed ¹⁰O incorporation at m/e 616 (M + 2) and m/e 618 (M + 4). Similarly, the mass spectrum of phycocyanobilin dimethyl ester showed ¹⁰O incorporation at m/e 616 (M + 2) and m/e 618 (M + 4).

### Table II

Calculation of ¹⁸O incorporation from mass spectra for Experiment 3 in Table III

<table>
<thead>
<tr>
<th>m/z Column</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 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>644</td>
<td>614</td>
<td>0.0</td>
<td>100</td>
<td>100</td>
<td>89.6</td>
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<td>99.6</td>
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<td>618</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>89.6</td>
<td>89.6</td>
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<tr>
<td>647</td>
<td>616</td>
<td>13.0</td>
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<td>89.6</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
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<tr>
<td>648</td>
<td>618</td>
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<td>89.6</td>
<td>89.6</td>
</tr>
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<td>646</td>
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<td>0</td>
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</tr>
</tbody>
</table>

### Table III

¹⁸O incorporation into phycocyanobilin synthesized in cells incubated in a closed flask containing air enriched with ¹⁸O₂

Phycocyanobilin was cleaved from phycobiliproteins in boiling methanol, purified as the dimethyl ester and dimethyl ester-methanol (covalent) adduct, and analyzed in the mass spectrometer.

<table>
<thead>
<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 %</th>
</tr>
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<tr>
<td>Control</td>
<td>Phycocyanobilin dimethyl ester</td>
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<td>Phycocyanobilin dimethyl ester</td>
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<td>10.4</td>
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<td>99.6</td>
<td>99.6</td>
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<tr>
<td>2</td>
<td>Phycocyanobilin dimethyl ester</td>
<td>618</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>89.6</td>
<td>99.6</td>
<td>99.6</td>
</tr>
<tr>
<td>3</td>
<td>Phycocyanobilin dimethyl ester-methanol (covalent) adduct</td>
<td>644</td>
<td>13.0</td>
<td>100</td>
<td>89.6</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
</tr>
</tbody>
</table>

Subtracting the small contributions yields the residuals shown in Column 7. As with the residual at m/e 647 in Column 7, the very small residual at m/e 648 in Column 7 may be real or may simply represent the error in the calculation. Assuming the former, this species will contribute a naturally abundant isotope peak at m/e 648 as shown in Column 8. Subtracting Column 8 from Column 7 yields the true value of the peak at m/e 648 corresponding to ¹⁰O incorporation (Column 9). Column 10 summarizes the true ¹⁰O incorporations and in Column 11, they are shown normalized in terms of mole per cent of phycocyanobilin dimethyl ester-methanol (covalent) adduct at the three mass numbers. These are the parameters that are used in subsequent analyses.

- ¹⁰O incorporation at M + 2 and M + 4 for the double hydrolytic, the hydrolytic, and 1-molecule mechanisms is self-evident. Predicted incorporation for the 2-molecule mechanisms, in Experiment 1, for example, was calculated as follows: final ¹⁸O₂ in gas phase of flask containing algae = 10.4 atom %. The probability of incorporating 2 atoms of ¹⁰O into the lactam oxygen atoms of phycocyanobilin is 0.895 x 0.104 x 100 = 90.3%. The probability of incorporating 2 atoms of ¹⁰O is 0.104 x 100 = 11.7%. The probability of incorporating 1 atom of ¹⁰O and 1 atom of ¹⁰O is (0.895 x 0.104) + (0.896 x 0.104) x 100 = 18.6%.
Mechanism of Phycocyanobilin Synthesis

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 pO₂ and percentage of °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 pO₂ demonstrates that the cells respire prior to the time that significant quantities of chlorophyll a and phycobiliproteins are produced. During this time, the percentage of °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 pO₂ value passed through a minimum and began to increase again, suggesting the onset of photosynthesis and the production of oxygen (i.e. °°₂) from water. Corroboratively, the °O/°O ratio decreased sharply at this stage. Fig. 5 also shows that after about 24 h, the partial pressure of °O remained relatively constant, demonstrating the cessation of respiration. Finally, the pO₂ 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 °O.

Initially, we were surprised to observe °O incorporation into phycocyanobilin synthesized from δ-aminolevulinic acid and excreted from cells incubated with H₂°O. The absence of °O in excreted phycocyanobilin would have confirmed the experiments performed under °O 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 H₂°O at 37°C for 5 days at pH 1.7.

The observation of °O incorporation from H₂°O has important consequences with respect to experiments demonstrating °O incorporation into phycocyanobilin bound to apoprotein in vivo. There are three explanations for the incorporation of isotopically labeled oxygen from H₂°O into the excreted phycocyanobilin. First, incorporation of °O from solvent water could indicate the double hydrolytic mechanism or the hydrolytic mechanism for phycocyanobilin synthesis. However, incorporation of °O from water at M + 4 is not predicted by the hydrolytic mechanism. This, together with incorporation of °O₂ 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 °O per carboxyl group on average. Had exchange occurred between H₂°O and the —CO₂ 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 CH₃— group. This would mean that the maximum °O level in phycocyanobilin (assuming complete exchange) would be one-half that of labeled water. The observed °O incorporation from H₂°O into phycocyanobilin was two-thirds of that of labeled water (Table IV). That is, °O in phycocyanobilin was °O₂ (1.7 + 2 x 1.1) = 9.7, whereas the °O level in water was 15 atom %. This would seem to exclude the possibility that °O in the excreted phycocyanobilin can be ascribed to oxygen exchange with solvent water (see "Discussion").

**TABLE IV**

<table>
<thead>
<tr>
<th>Bile pigment</th>
<th>m/e</th>
<th>Peak</th>
<th>Size of peak (M + 2)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phycocyanobilin dimethyl</td>
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<td>M + 2</td>
<td>17.2</td>
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<td>Phycocyanobilin (covalent) adduct</td>
<td>648</td>
<td>M + 4</td>
<td>1.1</td>
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</table>

**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 pO₂ and percentage of °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 pO₂ demonstrates that the cells respire prior to the time that significant quantities of chlorophyll a and phycobiliproteins are produced. During this time, the percentage of °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 pO₂ value passed through a minimum and began to increase again, suggesting the onset of photosynthesis and the production of oxygen (i.e. °°₂) from water. Corroboratively, the °O/°O ratio decreased sharply at this stage. Fig. 5 also shows that after about 24 h, the partial pressure of °O remained relatively constant, demonstrating the cessation of respiration. Finally, the pO₂ 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 °O.

Initially, we were surprised to observe °O incorporation into phycocyanobilin synthesized from δ-aminolevulinic acid and excreted from cells incubated with H₂°O. The absence of °O in excreted phycocyanobilin would have confirmed the experiments performed under °O 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 H₂°O at 37°C for 5 days at pH 1.7.

The observation of °O incorporation from H₂°O has important consequences with respect to experiments demonstrating °O incorporation into phycocyanobilin bound to apoprotein in vivo. There are three explanations for the incorporation of isotopically labeled oxygen from H₂°O into the excreted phycocyanobilin. First, incorporation of °O from solvent water could indicate the double hydrolytic mechanism or the hydrolytic mechanism for phycocyanobilin synthesis. However, incorporation of °O from water at M + 4 is not predicted by the hydrolytic mechanism. This, together with incorporation of °O₂ 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 °O per carboxyl group on average. Had exchange occurred between H₂°O and the —CO₂ 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 CH₃— group. This would mean that the maximum °O level in phycocyanobilin (assuming complete exchange) would be one-half that of labeled water. The observed °O incorporation from H₂°O into phycocyanobilin was two-thirds of that of labeled water (Table IV). That is, °O in phycocyanobilin was °O₂ (1.7 + 2 x 1.1) = 9.7, whereas the °O level in water was 15 atom %. This would seem to exclude the possibility that °O in the excreted phycocyanobilin can be ascribed to oxygen exchange with solvent water (see "Discussion").
Mechanism of Phycocyanobilin Synthesis

\[
m/e 644, (0.903 \times 0.903 \times 100) = 81.5\%; m/e 646, (2 \times 0.903 \times 0.907 \times 100) = 17.5\%; m/e 648, (0.907 \times 0.907 \times 100) = 0.9\%.
\]

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 \(^{18}O\) into phycocyanobilin attached to apoprotein in the \(C.\ caldarium\) chloroplast. This exchange would be small on the basis of the experiment done with \(H_2\)\(^{18}O\) because the incubation times in experiments done under \(^{18}O\) 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 \(^{18}O\) 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 then the minimum \(^{18}O\) level in the gas phase at the end of the experiment. For example, in Experiment 1, the final \(^{18}O\) level in the gas phase was 10.4%. Since the \(^{18}O\) in phycocyanobilin dimethyl ester was 9.3%, this would suggest a minimum exchange of 10.4 - 9.3/10.4 \times 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 \(^{18}O\) 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 \(H_2\)\(^{18}O\).

The question arises whether the exchange reaction costs 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 \(M + 4\) species, eventually to zero when exchange is complete. For \(M + 2\), the apparent incorporation would initially rise from zero, then 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, \((dm/dt) = -kn\) where \(k\) is the rate constant for exchange, and \((dn/dt) = kn - kn\) where \(k\) is the rate constant for exchange. The solution to these differential equations is:

\[
m = m_0 e^{-kt} \quad \text{and} \quad n = kn_0 e^{-kt}
\]

Differentiating with respect to time, it can be shown that \(n\) is maximum when \(t = 1/k\), i.e. \(m = n = m_0/e\).

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 \(^{18}O\) 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% \(^{18}O\) in the gas phase would predict \(M + 2 = 18.6\%\) and \(M + 4 = 1.1\%\). 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% \(^{18}O\) in the gas phase) was 18.7%, the exchange is 18.7 - 16.5/18.7 \times 100 = 12%. This exchange is reasonable on the basis of the exchange observed in the experiment with \(H_2\)\(^{18}O\). 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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