Protoheme Turnover and Chlorophyll Synthesis in Greening Barley Tissue

Paul A. Castelfranco and Owen T. G. Jones

Department of Biochemistry, University of Bristol, Bristol, BS8 1TD, England

ABSTRACT

Studies in which 14C-labeled precursors were fed to etiolated barley leaves (Hordeum vulgare L. var. Proctor) yielded chlorophyll and protoheme having similar specific radioactivities. These findings indicate: (a) there appears to be a rapid turnover of protoheme in the absence of net synthesis; (b) both pigments probably originate from a single 5-aminolevulinic acid pool; (c) the efficient utilization of glutamate-1-14C and the relatively poor utilization of glycine-2-14C suggest that 5-aminolevulinic acid is probably synthesized by a pathway other than 5-aminolevulinic acid synthetase (succinyl CoA-levulinic acid sucrinyltransferase) in agreement with previously published work; (d) protoheme turnover appears to be faster under conditions which allow for rapid chlorophyll accumulation; (e) difference spectra indicate that mitochondrial cytochromes make a relatively minor contribution to the total heme in barley leaves. These findings are discussed in the light of current knowledge about tetrapyrrole regulation in photosynthetic organisms.

Recent findings suggest that during Chl biosynthesis in higher plants ALA1 may not be formed by the succinyl CoA levulinic acid synthetase reaction (EC 2.3.1.37) (3, 5). The most effective precursors of radioactive ALA were not labeled succinate and glycine-2 14C, but the 5-carbon dicarboxylic compounds, 2-ketogluutaric acid, glutamic acid, and glutamine. A pathway in which the whole 5-carbon skeleton of these compounds is converted to the 5-carbon skeleton of ALA has been postulated (5).

Higher plants synthesize two classes of tetrapyrrole pigments (Chl and hemes) and contain two types of organelle (chloroplasts and mitochondria), that have some limited biosynthetic autonomy. It cannot be decided a priori whether all higher plant ALA is formed via a single biochemical sequence or whether it is formed by either of two pathways, namely, the succinyl transfer reaction or the newly postulated 5-carbon pathway, depending on the tissue or the organelle in question and the identity of the tetrapyrrole that is being made. It is of great interest to ascertain whether the postulated 5-carbon pathway can also be responsible for the synthesis of heme in (a) photosynthetic tissues and (b) nonphotosynthetic tissues of higher plants.

In the present study we have investigated heme biosynthesis in a developing photosynthetic tissue: etiolated barley seedlings during the first 5 hr of illumination. A parallel study is being planned, utilizing a nonphotosynthetic tissue which is undergoing rapid development of mitochondrial respiration, such as aging potato tuber disks (8).

MATERIALS AND METHODS

Materials. MN Kieselgur G and MN Kieselgel G were obtained from Macherey Nagel and Co.; Whatman cellulose powder, microgranular form, from W. and R. Balston Ltd.; AG 50W-X8 from Bio-Rad Laboratories, Richmond, Calif.; 2,6-Di-tet-butyl-p-cresol from B. D. H. Chemicals Ltd., Poole, Dorset, England; 5-aminolevulinic acid from Koch-Light Laboratories Colnbrook, Bucks, England. Diethyl ether was shaken with 5% FeSO4 (w/v) in 1% H2SO4 (v/v), washed with H2O and dried over anhydrous Na2SO4. It was stored at 4°C in the dark. Other solvents were analytical reagent grade and were used without further purification. Crystalline protoheme IX was prepared from human blood (11).

The following radioactive substrates were obtained from The Radiochemical Center, Amersham, Bucks, England: sodium bicarbonate-14C, 59.6 mCi/mmol; L-glutamic acid-14C(U), 265 mCi/mmol; DL-3-[1-14C]glutamic acid, 29 mCi/mmol; glycine-1-14C, 55.1 mCi/mmol.; glycine-2-14C, 51 mCi/mmol; 5-aminolevulinic acid hydrochloride, 53 mCi/mmol; FeCl3, 4.8 mCi/μmol initial specific radioactivity.

Plant Material. Barley seeds (Hordeum vulgare L. var. Proctor) were soaked overnight in tap water and germinated in the dark for 7 days in H2O-saturated peat soil at 25°C.

Incubation Conditions. The etiolated primary leaves were cut to a length of 6 cm from the tip. Five g samples of leaf tissue were placed with the cut end dipping into 5 ml of H2O contained in a 25-ml beaker. An air current from a fan placed 30 cm from the plant material facilitated the uptake of the H2O and dissolving materials by enhancing transpiration. Illumination was 0.87 mmol/cm² from a cool white fluorescent source. The incubation was carried out for 5 hr at 22 to 25°C; after 4 hr, if all the solution was taken up, 0.5 ml of H2O was added to prevent desiccation of the plant material.

Normally, when radioactive substrates were used, about 5 μCi of the substrate were dissolved in the 5 ml of H2O. In the 14CO2 feeding experiment the barley tissue was placed in a vacuum desiccator (4.6 liters total capacity) which contained also, in a separate beaker, 100 μCi of Na214CO3. The radioactive gas was liberated by careful addition of 2 ml lactic acid to the

1 This research was made possible by a fellowship award from the John Simon Guggenheim Memorial Foundation to one of us (P.A.C.). The financial support of the Science Research Council of Great Britain is gratefully acknowledged.
2 Permanent address: Department of Botany, University of California, Davis, California 95616.
3 Abbreviation: ALA: 5-aminolevulinic acid.
Na\textsuperscript{14}CO\textsubscript{2} solution. The desiccator was placed underneath the cool white light source which was shielded by a 5-cm layer of H\textsubscript{2}O to decrease the heating effect. At the end of the incubation, the desiccator’s atmosphere was exhausted through a Ba(OH)\textsubscript{2} trap before the lid was lifted. Trace amounts of FeCl\textsubscript{3} were dissolved in the 5 ml of H\textsubscript{2}O to which 50 \mu moles of ascorbic acid were added to keep Fe(OH)\textsubscript{3} from precipitating. No carrier FeCl\textsubscript{3} was added.

**EXTRACTION OF PIGMENTS**

**Chlorophyll Extraction.** The leaves were cut into 2-cm segments, placed in a mortar, and ground in liquid N\textsubscript{2}. The frozen powder was extracted three times with 33.3 ml of ice cold ammoniacal acetone. This solution consisted of 10 ml of 1\% NH\textsubscript{4}OH (v/v) 100 mg of 2,6-Di-\textit{tert}-butyl-p-cresol, and 90 ml of acetone. The three extracts were pooled and set aside for Chl analysis.

**Protoheme Extraction.** Ice cold ammoniacal acetone was found not to extract any protoheme. The solid residue after Chl extraction was stirred twice with 25 ml of 2\% concentrated HCl in acetone (v/v) to extract the protoheme. A third treatment with acid acetone failed to extract any more protoheme—although, as one might expect, this solid residue still contained heme c. The final residue was discarded. A 1/10 aliquot of the combined acid acetone extract was carefully evaporated to dryness in a Büchi rotary evaporator. The residue was redissolved in acetone and evaporated again to remove any trace of HCl. The protoheme present in the residue was estimated by its pyridine hemochrome difference spectrum.

**Porphyrin Extraction.** Porphyrin analysis was performed on both the acetone-NH\textsubscript{4}OH and the acetone-HCl extracts. The ammoniacal acetone was mixed with 1 volume of petroleum ether (b.p. 40–60 C), the aqueous acetone phase was withdrawn and the petroleum ether was washed with 1 volume of 80\% methyl alcohol. The acetone and methanol phases were combined and evaporated almost to dryness. Twenty ml of H\textsubscript{2}O were added and the cloudy suspension was adjusted to pH 4 by careful addition of acetic acid. The porphyrins were drawn into ether by repeated extraction. The ether, about 330 ml total volume, was washed with H\textsubscript{2}O and concentrated to 30 ml. This concentrated ether was washed repeatedly with 0.1\% HCl (w/v) to extract coproporphyrin, followed by 3\% HCl (w/v) to extract protoporphyrin (11).

The acetone-HCl extract was mixed with ether. Then, H\textsubscript{2}O was added to cause the two phases to separate: the lower phase was adjusted to pH 4.0 with sodium acetate, and the ether layer was washed well with H\textsubscript{2}O. The ether was extracted successively with 0.1\% (w/v), and 3\% HCl (w/v) to remove coproporphyrin and protoporphyrin, respectively (11). The extraction was followed by monitoring porphyrin fluorescence with a 3650 \AA\ UV lamp. The porphyrins were determined spectrophotometrically (2).

**CHLOROPHYLL PURIFICATION BY SOLVENT PARTITION**

The total Chl extract (approximately 100 ml of ammoniacal acetone) was mixed with 50 ml of petroleum ether (b.p. 40–60 C) in a separating funnel. The yellow lower phase was discarded. The green upper phase was washed four times with 50 ml of ice cold MgCO\textsubscript{3}-saturated H\textsubscript{2}O, which was added dropwise through a fluted paper filter to avoid emulsions. The petroleum ether was then washed four times with 50 ml of ice cold 80\% methyl alcohol and evaporated to dryness in the rotary evaporator. The residue was redissolved in 1 ml of cyclohexane and transferred to TLC plates.

**CHROMATOGRAPHY OF CHLOROPHYLL AND Porphorhbide a**

Chlorophyll a was purified by two consecutive chromatographic steps. Twenty-four g of Kieselgur, 6 g of Kieselgel, 6 g of powdered analytical reagent grade CaCO\textsubscript{3}, and 36 mg of Ca(OH)\textsubscript{2} were shaken vigorously with 69 ml of 5 mm K\textsubscript{2} ascorbate buffer, pH 7.1 to 7.3 (W. T. Griffiths, private communication), and the gel was spread on five 20 cm × 20 cm plates to an approximate thickness of 0.5 mm. The plates were air-dried and activated by heating to 80 C for 2 hr. The plates were developed with petroleum ether (b.p. 60–80 C)-isopropyl alcohol-H\textsubscript{2}O (100:5:0.25). Chl a (R\textsubscript{f}, about 0.7) is separated from pheophytin a and Chl b. The Chl a band was scraped, eluted with ice cold ether and the extract evaporated to dryness. The Chl a residue corresponding to 5 g of leaf tissue was dissolved in 1 ml of cyclohexane and transferred to cellulose plates.

Forty-five g of powdered cellulose were shaken vigorously with 100 ml of H\textsubscript{2}O and spread upon five 20 cm × 20 cm plates to an approximate thickness of 0.5 mm. The plates were air-dried and activated by heating to 80 C for 2 hr. The plates were developed with petroleum ether (b.p. 60–80 C)-acetone-n-propyl alcohol (90:10:0.45) (20). The Chl a band was eluted with ice cold ether and the eluate evaporated to dryness. The Chl a residue corresponding to 5 g of leaf tissue was redissolved in 5 ml of ether and converted to pheophorbide a, which was purified by partition between ether and HCl (17). The pheophorbide-containing ether was evaporated to dryness: the residue was dissolved in methyl alcohol and spotted on cellulose plates which were developed with petroleum ether (b.p. 60–80 C)-acetone-n-propyl alcohol (80:20:0.45). Chromatography of pheophorbide a yielded three bands which were located by their pink fluorescence when viewed under a 3650 Å UV lamp: a main band (R\textsubscript{f}, about 0.5), a second band which stayed at the origin, and a very faint band which moved right behind the main band. Only the main band was collected by scraping the gel and eluting with methyl alcohol, although the band at the origin was spectrophotometrically identical to the main band, as shown also by other workers with other chromatographic systems (17). The methyl alcohol extract was evaporated to dryness and the residue dissolved in 5 ml of ether for spectrophotometric and radioactivity measurements. All chromatography was carried out in the dark at 12 C. Solvent evaporation was done in a Büchi rotary evaporator avoiding overheating. In all manipulations the pigments were shielded from direct light.

**PURIFICATION OF HEME FROM BARLEY EXTRACTS**

**Crystallization of Protoheme.** After aliquots of the acetone-HCl extract had been assayed for protoheme, the remaining extract was taken to dryness and the residue dissolved in 3 ml of glacial acetic acid. A solution containing about 5 mg of carrier protoheme in 15 ml of acetone-glacial acid (3:1, v/v containing 0.5% SrCl\textsubscript{2}) was added (11). The exact concentration of carrier protoheme was determined by the pyridine hemochrome assay. The volume of heme solution was then reduced by careful evaporation until fine protoheme crystals just began to form. A few drops of glacial acid were added, and the flask was allowed to stand overnight in the dark. The protoheme crystals were collected by centrifugation, washed repeatedly with H\textsubscript{2}O, dried in vacuo, and then washed repeatedly with ether. To calculate the recovery of protoheme, the crystals were dissolved in 2 ml of pyridine and small aliquots were removed for the pyridine hemochrome assay. When necessary, aliquots of the pyridine solutions were dried on planchetteS and counted using a gas flow counter.
Solvent Partition. For the study of $^{56}$FeCl$_3$ incorporation into protoheme, after aliquots of the acetone-HCl extract had been assayed for protoheme, 5 ml of heme carrier solution in acetone were added to the remainder. The acetone extract (50 ml) was mixed with an equal volume of ether and washed 5 times with 50 ml of 1% HCl (v/v) and twice with 50 ml of H$_2$O. The ether layer was evaporated to dryness, the residue dissolved in pyridine, and the specific radioactivity of the protoheme was determined.

SPECTROPHOTOMETRIC MEASUREMENTS

Chlorophyll and protochlorophyll were determined from the absorbances at 626, 645, and 663 nm (1, 4), and phophorhile $a$ from the absorbance of its $a$ peak at 667 nm ($\epsilon$ mm = 55.5) (15). Protoheme was determined by its pyridine hemochrome difference spectrum. Protoheme-containing samples were dissolved in 5 ml of alkaline pyridine (2 volumes pyridine-3 volumes 0.2 M KOH) and the solution was divided equally between the two cuvettes of a split beam spectrophotometer. Na$_2$SO$_4$ was added to the sample cuvette, and the difference spectrum of the reduced minus oxidized pyridine hemochrome was recorded. The concentration of protoheme was calculated using the $\Delta \epsilon$ mm = 20.7, where $\Delta \epsilon$ represents the difference in extinction between the $\alpha$ band at 557 nm and the trough at 540 nm, between the $\alpha$ and $\beta$ peaks (19). For plant extracts it was usually necessary to work with a sensitivity such that 0.1 absorbance unit gave full scale deflection on the spectrophotometer recorder.

Coproporphyrin was determined in 0.1% HCl (w/v) and protoporphyrin in 3% HCl (w/v), from the absorbances of their Soret peaks (2, 11).

Spectra were recorded on a split-beam spectrophotometer (9) employing a Hilger D330 monochromator. The reciprocal dispersion was 25 A/mm and the slit widths were kept at 0.15 mm.

Radioactive Measurements. Radioactivity was measured with a gas-flow counter equipped with a Nuclear Chicago D-47 counting tube.

FREE AMINO ACID ANALYSIS

Five g of etiolated barley tissue were ground in liquid N$_2$. The frozen powder was suspended in 20 ml of absolute ethyl alcohol and boiled for 10 min. The suspension was cooled, centrifuged, and the residue was extracted again with 30 ml of boiling 80% ethyl alcohol. The two extracts were combined and partitioned with 50 ml of chloroform and 50 ml of H$_2$O. The chloroform was discarded and the aqueous layer was washed once with 50 ml of chloroform and then with 50 ml of ether. The aqueous phase was passed through a column of AG 50W-X8 charged with H$.^+$ The column was eluted with 20% NH$_4$OH (v/v) and the eluate was evaporated to dryness under reduced pressure. The amino acids present in the residue were determined by means of an automatic amino acid analyzer with reference to standards.

RESULTS

On continuous illumination, the Chl content of dark-grown barley tissue rises rapidly, reaching within 12 hr a value 100 times greater than the phototransformable protochlorophyll present in the etiolated tissue (Fig. 1). On the other hand, during this time acid acetone-extractable protoheme increases at most by one-third the original value (Fig. 1).

Table I summarizes a series of experiments on the simultaneous labeling of Chl $a$ and protoheme from a variety of $^{14}$C-

![Fig. 1. Chlorophyll (O) and protoheme (●) accumulation in etiolated barley tissue as a function of illumination time.](image)

<table>
<thead>
<tr>
<th>Labeled Precursor</th>
<th>Uptake</th>
<th>Specific Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm X $10^3$/g</td>
<td>cpm/mmol</td>
</tr>
<tr>
<td>$^{14}$CO$_2$</td>
<td>62.9</td>
<td>71.5</td>
</tr>
<tr>
<td>Glycine-1-$^{14}$C</td>
<td>7.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Glycine-2-$^{14}$C</td>
<td>11.9</td>
<td>17.8</td>
</tr>
<tr>
<td>DL-Glutamate-1-$^{14}$C</td>
<td>8.2</td>
<td>174</td>
</tr>
<tr>
<td>L-Glutamate-U-$^{14}$C</td>
<td>11.5</td>
<td>67.4</td>
</tr>
<tr>
<td>ALA-4-$^{14}$C</td>
<td>6.8</td>
<td>1460</td>
</tr>
</tbody>
</table>

precursors. Protoheme and phenophorhile $a$ (derived from Chl $a$) were purified as described, and the specific radioactivity of each compound was determined. In each of these experiments using a single $^{14}$C-labeled precursor, the specific radioactivities in protoheme and phenohorhile $a$ were found to be very similar (Table I). Comparisons between different experiments (i.e., comparisons of specific radioactivities obtained from different $^{14}$C-labeled substrates) are more hazardous since the plant material and the incubation conditions varied considerably from one experiment to the next.

The effectiveness of different $^{14}$C compounds as precursors of labeled protoheme was compared directly by carrying out parallel incubations with different $^{14}$C substrates (Table II). Glutamate is definitely more effective than glycine in the la-
Table II. Labeling of Protoheme by Various Radioactive Compounds

Five g of etiolated barley leaves were incubated for 5 hr under fluorescent illumination, the cut end dipped in 5 ml of H$_2$O containing trace amounts of the radioactive precursor. In both experiments I and II the tissue samples were cut from the same tray and incubated with the various substrates simultaneously, under identical conditions.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Labeled Precursor</th>
<th>Specific Radioactivity</th>
<th>Uptake</th>
<th>Protoheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Glycine-1-^{14}C</td>
<td>9.4</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Glycine-2-^{14}C</td>
<td>9.0</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>DL-Glutamate-1-^{14}C</td>
<td>9.8</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>L-Glutamate-U-^{14}C</td>
<td>10.1</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>DL-Glutamate-1-^{14}C</td>
<td>8.2</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>L-Glutamate-U-^{14}C</td>
<td>6.9</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>ALA-4-^{14}C</td>
<td>6.8</td>
<td>1240</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>$^{59}$FeCl$_3$</td>
<td>6.1</td>
<td>1090</td>
<td></td>
</tr>
</tbody>
</table>

In Figure 1 it was shown that there was little increase in extractable protoheme during greening. Spectra of whole leaf homogenate at 77 K confirmed that there was no massive development of b-type Cyt during greening. Such spectra also showed that Cyt oxidase (absorbing at 599 nm at 77 K) was a very minor component of the total heme spectrum (Fig. 2), indicating that mitochondrial Cyt are present in relatively small amounts. The main heme proteins present appear to be those of the etioplast: Cyt f (absorbing at 553 nm), b 559, and b 563.

Five-g samples of barley tissue were incubated with either 5 ml of 20 mM ALA or 5 ml of H$_2$O. After 5 hr, Chl, protoheme, and porphyrins were extracted from both tissue samples. The light incubation and the dark incubation were carried out on separate days with separate batches of tissue. In the light, the ALA treatment caused a definite increase in Chl a and a lesser increase in Chl b; protoclorophyllide and free porphyrins (mostly protoporphyrin) accumulated in the ALA-treated tissue. In the dark, the ALA-treated tissue contained large amounts of protoclorophyllide, a considerable amount of protoporphyrin, and a trace of coproporphyrin. The control tissue contained only a normal amount of protoclorophyllide (Table III). No uroporphyrin was detected in either light or dark experiments. The difference in protoheme levels between the two experiments (6.8 and 6.6 for the light experiment: 3.9 and

![Fig. 2. Reduced minus oxidized difference spectra at 77 K of an acetone powder of etiolated barley tissue. Acetone powder (100 mg) was suspended in 5 ml of 50 mM HEPES buffer, pH 7.5, containing 20% sucrose. The sample was reduced with dithionite and the reference cuvette was oxidized with ferricyanide. Light path of cuvette was 2 mm.](image)

Table III. Effect of Exogenous ALA on the Accumulation of Various Tetrapyrroles

Five g of etiolated barley leaves were incubated for 5 hr, the cut end dipped in either 20 mM ALA or H$_2$O. The light and dark incubations were carried out in two separate experiments. Abbreviations: Chl, Chlorophyll; P.chl$d$, protoclorophyllide; Proto, protoporphyrin; Copro, coproporphyrin; Heme, protoheme.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Chl a</th>
<th>Chl b</th>
<th>P.chl$d$</th>
<th>Proto</th>
<th>Copro</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>ALA</td>
<td>182.6</td>
<td>48.8</td>
<td>17.4</td>
<td>9.6</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>150</td>
<td>45.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dark</td>
<td>ALA</td>
<td>0</td>
<td>113</td>
<td>11.2</td>
<td>trace</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>0</td>
<td>10.6</td>
<td>0</td>
<td>0</td>
<td>4.2</td>
</tr>
</tbody>
</table>
4.2 for the dark experiment) is within the normal variation for different tissue batches. The significant point in this experiment is that the ALA treatment, which caused a large accumulation of protoporphyrin IX, failed to cause any increase in extractable heme, either in the light or in the dark (Table III).

**DISCUSSION**

Our experimental findings show a good correlation between the labeling of heme and that of Chl from exogenous $^{14}C$ compounds (Tables I and II). Both of these tetrapyrroles are labeled more effectively from glutamate than glycine, in agreement with the 5-carbon pathway previously postulated on the basis of labeling studies on ALA accumulated in levinic acid-treated tissues (5). The specific activities in protoheme and Chl are in most cases sufficiently close to suggest that both pigments are synthesized from a common ALA pool (Table I). This closeness implies that the regulation of Chl and hemes does not involve spatial compartmentation at the level of ALA production.

The Chl level undergoes a 35-fold increase during the first 5 hr of illumination, whereas the heme level remains unchanged (Fig. 1). To explain the labeling of heme in the absence of net synthesis we might suggest as a working hypothesis that while Chl is being synthesized, acid-extractable protoheme is turning over at a comparable rate. Heme turnover has been demonstrated, directly or indirectly, to occur in several hemoprotein systems. Bunn and Jandl (7) have shown the exchange of ferriheme between hemoglobins $\alpha$ and $\beta$. Bock and Siekewitz (6) have studied the independent turnover of heme and apoprotein in rat liver microsomal Cyt $b_6$, using in vivo double labeling techniques. Haddad (12) has shown that an $E. coli$ mutant which lacks ALA synthetase can nevertheless synthesize Cyt $b$ apoprotein and embed it into the cytoplasmic membrane. Electron transport particles prepared from this mutant lack NADH oxidase activity, which can be introduced by incubating them in vitro with hematin and ATP.

Unfortunately, our experimental technique, involving the feeding of labeled substrates through the cut surface of barley leaves, does not lend itself to pulse-chase experiments, which are required to confirm our protoheme turnover hypothesis and to give a quantitative description of the turnover kinetics. We are indeed considering such experiments using biological material with a greater absorptive surface to volume ratio than barley leaves.

The heme proteins present in etiolated barley tissue appear to be primarily those associated with the photosynthetic apparatus: Cyt $f$, $b$ 599, and $b$ 563 (Fig. 2). The very slight Cyt oxidase peak at 599 nm indicates that the mitochondrial contribution to the total tissue hemes was minimal. The heme protein spectrum was not significantly different after the tissue was exposed to light for 5 hr. Acetone containing 2% HCl would be expected to dissociate protoheme from Cyt $b$ 559 and $b$ 563, but not from Cyt $f$. It has been shown that in barley seedlings the level of Cyt $b$ 563 does not change during greening; Cyt $b$ 559 does change from a low potential to a high potential form, but the total level remains the same (18). Because of the low level of mitochondrial Cyt in this tissue, our experiments cannot give us any information concerning the fate of mitochondrial heme.

Our experiments indicate that heme labeling is greater under those conditions that favor Chl synthesis; i.e., it is lower in the dark and when the tissue has already accumulated a near maximal Chl level. It should be pointed out that if protoheme labeling is caused by turnover, a 50% decrease in the specific radioactivity of protoheme after 5 hr exposure could represent a decrease in turnover rate much greater than 50%.

Incubation of seedlings with 20 mm ALA (Table III) caused a rise of Chl in the light and an accumulation of protochlorophyllide and protoporphyrin in both light and dark. The level of protoheme, however, was not affected. That its sizable protoporphyrin accumulation was not reflected in a rise of protoheme suggests either that Fe chelation is involved, directly or indirectly, in the control of heme synthesis, or that a mechanism exists for removing excess protoheme.

Granick and Sassa (14) have reviewed the control of heme and Chl synthesis in a variety of biological systems, focusing in particular on hepatic microsomal hydroxylases, hemoglobin synthesis, heme and bacteriochlorophyll synthesis in *R. spheroides*, and Chl synthesis in *Euglena* and higher plants. In general, it may be said that ALA availability is the limiting factor in all of these processes and therefore that the synthesis of ALA is the major control point. Heme seems to be involved in the control of ALA synthesis in a variety of ways: for instance, in cultured chick embryo liver cells, heme inhibits the synthesis of ALA synthetase at the translational level (possibly acting through a hemoprotein), but it may also affect the transport of ALA synthetase into the mitochondria. In *R. spheroides* heme acts as a repressor in the synthesis of ALA synthetase and also acts as a feedback inhibitor of the enzyme.

---

**Fig. 3.** Proposed scheme for the control of tetrapyrrole synthesis in higher plants. ALA; 5-aminolevulinic acid; Proto: protoporphyrin IX; P. chlide: protochlorophyllide; Chl: chlorophyll; Chl: chlorophyll; Heme: protoheme.
Photosynthetic bacteria form Chl only under low O₂ tension and show no light requirement for this synthesis. The O₂ control appears to be mediated at the Mg insertion step because the enzyme Mg chelatase is inhibited by O₂ (13). This inhibition causes a transient accumulation of protoporphyrin in aerobic environment. This protoporphyrin is diverted to protoheme, and when the requirement for respiratory Cyt is satisfied, the excess protoheme shifts off ALA synthesis (16). Higher plants form Chl under aerobic conditions, but require light in order to do so. Light is needed for accumulation of ALA in plant tissues treated with levulinic acid (4). It is not known how light exerts its control over ALA formation, but since the conversion of protoclophyllide to chlorophyllide is the only known light-requiring step in Chl synthesis, it is often assumed that the light control over the first step of this pathway must be mediated through protoclophyllide photoconversion.

Duggan and Gassman (10) have recently shown that iron chelators stimulate the biosynthesis of porphyrins in etiolated leaves, an effect that mimics that of the addition of exogenous ALA. These chelators may prevent heme synthesis by making iron unavailable. In agreement with our observations suggesting a rapid protoheme turnover, these authors report that the heme content of leaves incubated with dipyridyl is lower than that of untreated leaves. If we postulate that synthesis of ALA is regulated by protoheme, it is possible to construct a simple scheme for the control of ALA synthesis in higher plants which relates Duggan and Gassman's observations on the effect of chelators with our own observations suggesting protoheme turnover (Fig. 3).

In analogy to the accepted scheme for tetrapyrrole regulation in photosynthetic bacteria, the coarse control over the whole system is exerted by protoheme, possibly through a hemoprotein, on the synthesis of ALA. The fine control is entrusted to a heme breakdown mechanism which regulates the protoheme level.

Light can overcome the protoheme block on ALA synthesis. It is not known whether this light effect is tied to protoclophyllide photoconversion as indicated by the dotted line in Figure 3. Only a careful measurement of the action spectrum for ALA accumulation in the presence of levulinic acid can resolve this point. Teleologically, it would seem that mediating the light effect on ALA formation through protoclophyllide photoconversion is a good way to ensure that the needs of the Chl branch are immediately felt on the first step of the tetrapyrrole pathway.

Acknowledgment—We are grateful to Dr. B. Pickering and Mrs. H. Searle of the Anatomy Department at Bristol University for the amino acid analyses.

LITERATURE CITED