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Bile pigment synthesis in plants

Incorporation of haem into phycocyanobilin and phycobiliproteins in *Cyanidium caldarium*

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A procedure was developed whereby haem was taken up by dark-grown cells of the unicellular rhodophyte *Cyanidium caldarium*. These cells were subsequently incubated either in the dark with 5-aminolaevulinate, which results in excretion of phycocyanobilin into the suspending medium or incubated in the light, which results in synthesis and accumulation of phycocyanin and chlorophyll *a* within the cells. Phycocyanobilin was isolated from phycocyanin by cleavage from apoprotein in methanol. Phycocyanobilin prepared from phycocyanin or excreted from cells given 5-aminolaevulinate was methylated and purified by t.l.c. By using ^{14}C labelling either in the haem or in 5-aminolaevulinate administered, haem incorporation into phycocyanobilin was demonstrated in both dark and light systems. Since chlorophyll *a* synthesized in the light in the presence of labelled haem contained no radioactivity, it was clear that haem was directly incorporated into phycocyanobilin and not first converted into protoporphyrin IX. These results clearly demonstrate phycocyanobilin synthesis via haem and not via magnesium protoporphyrin IX as has also been postulated.

The formation of open-chain tetrapyrroles (bile pigments) in both plants and animals has been known for many years, although it is only comparatively recently that the mechanism of biosynthesis of these compounds has been studied in detail. In animals there is now abundant evidence (reviewed by Schmid & McDonagh, 1975) to show that bile pigments are derived from degradation of haem by the scheme shown in Fig. 1. For example, administration of ^{14}C-labelled haem to experimental animals results in incorporation of label into the bile pigments produced. In mammals the first bile pigment formed, biliverdin (Fig. 1), does not accumulate but is converted enzymically into bilirubin (Fig. 1), which is conjugated and excreted in bile (Schmid & McDonagh, 1975). The degradation of mammalian haem to biliverdin is catalysed by the microsomal enzyme haem oxygenase (EC 1.14.99.3; Tenhunen *et al.*, 1968). However, there appears to be no function for the animal bile pigments other than in providing a pathway for elimination of the haem derived from the turnover of haemoglobin and other haemoproteins.

Several plant pigments (phycobilins) have been identified with structures so closely related to biliverdin that they have also been termed bile pigments. The best known are phycocyanobilin (Fig. 1), which is the covalently-linked prosthetic group of the phycobiliproteins allophycocyanin and phycocyanin, and the closely related phycoerythrobilin, which is the covalently linked prosthetic group of phycoerythrin (O'Carra & O'hEocha, 1977). In contrast with the mammalian bile pigments, these compounds are metabolically functional occurring in red (*Rhodophyta*), blue–green (*Cyanophyta*) and cryptomonad (*Cryptophyta*) algae, where they act as accessory photosynthetic pigments. In addition all higher plants contain phytochrome, an important regulator of several fundamental processes, and this is also a bile-pigment–protein complex (Pratt, 1978).

Although until now no tetrapyrrolic precursor of phycobilins has been directly demonstrated, examination of the structures of phycocyanobilin and phycoerythrobilin suggests that their formation may utilize the classical porphyrin biosynthetic pathway. The structural similarity of plant and animal bile pigments is further evident in the fact that both are IXα isomers, i.e. the order of the side chains is that in type-IX porphyrins and both types of bile pigment lack the α-methene bridge of type-IX porphyrins.

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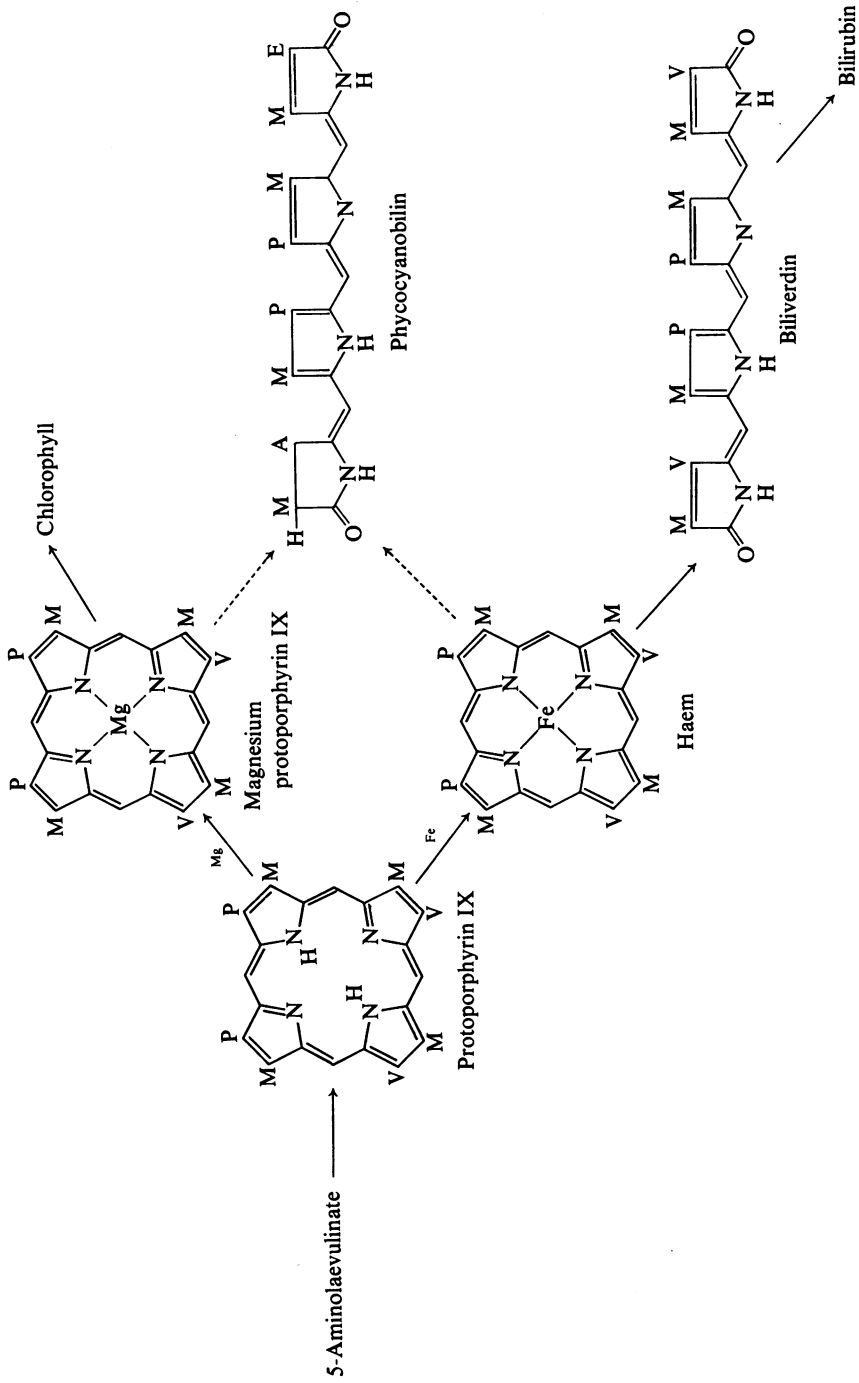

Fig. 1. *Metabolic pathways for synthesis of haem, chlorophyll and bile pigments*
The solid arrows represent known pathways; the broken arrows represent possible pathways for biosynthesis of phycocyanobilin from different metalloporphyrin precursors. Abbreviations used: M, $-CH_3$; P, $-CH_2CH_2CO_2H$; V, $-CH{=}CH_2$; E, $-CH_2CH_3$; A, $=CH-CH_3$.

This relationship led to suggestions that, like biliverdin, the carbon skeleton of phycobilins was derived from protoporphyrin IX (Bogorad & Troxler, 1967). These ideas received strong support when it was shown that ^{14}C-labelled 5-aminolaevulinate, a known precursor of protoporphyrin IX formation in animals, was incorporated into the phycocyanin of the rhodophyte *Cyanidium caldarium* (Troxler, 1972). Further experiments (Troxler, 1972), with this organism showed that formation of [^{14}C]phycocyanobilin was accompanied by the production of equimolar amounts of ^{14}CO as occurs in haem metabolism and the specific-activity ratio of the products was that expected if they were derived from a common porphyrin precursor.

Although these experiments proved that phycocyanobilin was formed via a porphyrin (presumed to be protoporphyrin IX), direct evidence on the ring-opening step has proved difficult to obtain. Degradation via haem was clearly a strong possibility (Bogorad & Troxler, 1967), but more recent work with model systems has suggested the feasibility of a magnesium-mediated pathway (Hudson & Smith, 1975), perhaps via magnesium protoporphyrin IX. This alternative has attractions since greening plants manufacture large amounts of magnesium protoporphyrin IX destined for chlorophyll and also because the reduced ring of phycocyanobilin (Fig. 1) suggests that its immediate precursor may be a chlorin rather than a porphyrin.

The mechanism of incorporation of molecular O_2 into the terminal lactam oxygen atoms of bile pigments has been studied by ^{18}O labelling methods. In living rats (Brown & King, 1978), in the haem oxygenase system (King & Brown, 1978) and in various chemical model systems (Chaney & Brown, 1978; Jackson *et al.*, 1978) degradation of haem occurred by a Two-Molecule mechanism, whereby the lactam oxygen atoms of the product bile pigment are derived from two different O_2 molecules. Recently, we have shown (Troxler *et al.*, 1979; Brown *et al.*, 1980) that biosynthesis of phycocyanobilin also involves a Two-Molecule mechanism and this was considered as evidence suggesting that phycocyanobilin was formed by haem degradation, although this was not proved since the mechanism of degradation of magnesium porphyrin derivatives is unknown.

The direct experiment of looking for [^{14}C]haem incorporation by algae has hitherto been considered impracticable because of the difficulty in uptake of haem by algal cells. This is especially true of cells of *C. caldarium*, which grow optimally at around pH 2 where haem is totally insoluble. In the present work we describe a procedure whereby this problem of uptake may be overcome and also ^{14}C labelling experiments, which thereby become possible. *C. caldarium* is a convenient organism for such experiments because it can be grown aerobically in the dark without producing pigment. The synthesis of phycocyanin and chlorophyll *a* may then be induced simply by exposure of cells to light. An equally attractive property of this organism is that when such dark-grown cells are incubated in acid medium with 5-aminolaevulinate in the dark, relatively large amounts of porphyrins and protein-free phycocyanobilin are excreted into the suspending medium (Troxler & Bogorad, 1966). This represents a very convenient system for study of phycocyanobilin synthesis.

Experimental

Materials

In general all materials obtained commercially were of analytical reagent grade. Solvents were redistilled before use. 5-Amino[4-^{14}C]laevulinic acid hydrochloride (sp. radioactivity 40μCi/mmol) and n-[^{14}C]hexadecane (863 d.p.m./μl) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. An additional sample of 5-amino-[4-^{14}C]laevulinic acid hydrochloride (sp. radioactivity 50 Ci/mol) was obtained from New England Nuclear, Boston, MA, U.S.A. BF_3 (14% in methanol) was purchased from BDH Chemicals, Poole, Dorset, U.K. Unlabelled 5-aminolaevulinic acid hydrochloride was obtained from Sigma (London) Chemical Co. and unlabelled haem from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

^{14}C-labelled haem was prepared by incubating reticulocyte-rich rabbit blood with 5-amino[4-^{14}C]-laevulinic acid. A high reticulocyte count was induced in a male New Zealand White rabbit by injections of a 2.5% (w/v) solution of phenylhydrazine on five consecutive days (Craig *et al.*, 1976). The rabbit was rested for 2 days and then bled by cardiac puncture under phenobarbitone anaesthesia. Amino[^{14}C]laevulinate was incubated with whole blood (1μCi/ml of blood) at 37°C for 24 h. Streptomycin and penicillin were added in concentrated solution to a final concentration of 0.4 mg/ml each. Haemin was crystallized by the method of Labbe & Nishida (1957). Whole blood was added to 12 vol. of an extraction mixture of acetone/strontium chloride in acetic acid. Protein precipitation was aided by brief boiling followed by stirring for 30 min. After removal of the precipitate by filtration, acetone was removed by heating to 100°C. Crystalline haemin, which formed on cooling, was collected and washed by filtration.

The specific radioactivity was determined as follows. A few crystals were dissolved in pyridine and a portion (50μl) was taken for liquid-scintillation counting of radioactivity. The concentration was determined by adding alkali and sodium dithionite to give the pyridine haemochrome, the absorbance being read at 557 nm ($\varepsilon = 32 \times 10^3$ litre·mol^{-1}·cm^{-1};

Falk, 1964). The radiochemical purity of ^{14}C-labelled haem was demonstrated by t.l.c. on Avicel cellulose plates developed with 2,6-lutidine/water (10:7, v/v) as described by Bissell *et al.* (1979). This procedure established that ^{14}C-labelled haem was not contaminated with 5-amino[^{14}C]laevulinate.

Organism

C. caldarium (Allen, 1959) is a unicellular organism that synthesizes chlorophyll *a*, allophycocyanin and phycocyanin in the light, but is unable to make these photosynthetic pigments when grown in the dark (allophycocyanin is a minor component, representing about 10% of the total phycobiliprotein; phycocyanin accounts for the remaining 90%). However, when cells grown heterotrophically in the dark are placed in the light in minimal medium minus glucose (pH 1.7) (Troxler, 1972), photosynthetic pigments are synthesized concomitantly with the development of a chloroplast in each algal cell (Bogorad *et al.*, 1963). Alternatively when dark-grown cells are resuspended in minimal medium (pH 1.7) containing glucose and 5-aminolaevulinate, relatively large quantities of porphyrin and protein-free phycocyanobilin are excreted into the suspending medium (Troxler & Bogorad, 1966). *C. caldarium* mutant III-D-2 was used in the present work since this strain produces more pigment per cell than does the wild-type. All cell cultures were prepared from a small inoculum grown in 1 litre of minimal medium supplemented with 1% (w/v) glucose (Allen, 1959) in 2-litre conical flasks on a rotary shaker at 37°C in the dark. These dark-grown cells were collected by centrifugation (15000 *g* for 15 min) and washed twice with water before use in experiments described below.

Haem uptake by C. caldarium

Dark-grown cells of *C. caldarium* were resuspended (1 ml of packed cells/10 ml) in a minimal medium such that the pH would permit solubility of haem. This was prepared by using the minimal medium of Allen (1959) as a basis, but omitting H_2SO_4, changing the concentration of KH_2PO_4 to 0.34 g/litre and adding $Na_2HPO_4,12H_2O$ to a concentration of 17.01 g/litre. This resulted in a medium at pH approx. 8. Concentrated haem solutions were prepared by dissolving a weighed quantity of haemin chloride in a minimum of 0.1 M-NaOH (less than 0.4 ml). This concentrated solution was added directly to the resuspended cells at pH 8 and the suspension was placed (in the dark) in a shaking incubator at 37°C. In trial experiments, samples were withdrawn periodically, the cells were removed by centrifugation (bench centrifuge) for 10 min and the haem absorbance was monitored at 384 nm. For haem incorporation experiments these cultures were maintained for 4–5 h to permit maximum haem uptake. After this period, cells were harvested by centrifugation (bench centrifuge), washed twice with water and resuspended in the appropriate acid medium (see below). During the washings, no haem was removed from the cells.

Formation of phycocyanobilin by C. caldarium in the dark

Dark-grown cells that had been exposed to pH 8 medium (with or without haem addition) as described above were resuspended in minimal medium (1 ml of packed cells/10 ml of medium) at pH 1.7 (Allen, 1959) containing 1.8% (w/v) glucose and 0.16% (w/v) 5-aminolaevulinic acid hydrochloride. The suspension was placed in a shaking incubator at 37°C for 90 h in the dark, during which time the system became very dark due to excretion of porphyrins and protein-free phycocyanobilin into the suspending medium. The cells were collected by centrifugation (bench centrifuge) for 10 min and the supernatant was extracted four times with chloroform (1.25 ml of chloroform/10 ml of medium). After centrifugation (bench centrifuge) for 10 min to eliminate any emulsion, the chloroform extract containing phycocyanobilin was taken to dryness under a stream of N_2.

Formation of phycocyanin and chlorophyll a by C. caldarium in the light

Dark-grown cells that had been exposed to pH 8 medium for 5 h (with or without haem addition) as described above were resuspended in minimal medium (1 ml of packed cells/10 ml of medium) at pH 1.7. The culture was placed in a conical flask in a shaking water bath at 37°C and illumination was supplied by two 40 W fluorescent tubes (approx. 5400 lx). After about 12 h greening was evident indicating phycocyanin and chlorophyll *a* synthesis and incubations were continued to a total of 64 h. The cells were collected by centrifugation (bench centrifuge) for 10 min and washed twice with water. The cells were resuspended in 0.1 M-phosphate buffer and disrupted by using a French pressure cell (Aminco American Instrument Co. Inc., Silver Springs, MD, U.S.A.) at 20000 lbf/in^2 (1.38×10^5 kPa) internal pressure. The homogenate was centrifuged (36000 *g* for 30 min) and the green supernatant made 50% saturated with $(NH_4)_2SO_4$. The precipitated material including phycocyanin and small quantities of allophycocyanin was redissolved in 1% (w/v) trichloroacetic acid and left overnight at room temperature. The precipitated denatured phycocyanin was collected by centrifugation (bench centrifuge, 10 min), and washed three times with water and three times with methanol. Phycocyanobilin was cleaved from phycocyanin by refluxing in boiling methanol for 16 h as previously described (Troxler *et al.*, 1978).

Esterification of phycocyanobilin

The phycocyanobilin prepared by either of the two methods described above was dissolved in 5ml of methanol and 5ml of 14% (w/v) BF_3 in methanol was added. After refluxing for 2min the solution was cooled to room temperature, 100ml of 1.0M-NaCl was added and the bile pigment diester was extracted into chloroform. The chloroform solution was washed with water, filtered through chloroform-moistened filter paper and evaporated to dryness under a stream of N_2.

Preparation of chlorophyll a

The pellet obtained from centrifugation after cell disruption (see above) was treated with 6vol. of acetone/water (5:1, v/v). After centrifugation (bench centrifuge) for 10min the green supernatant was retained and a second identical extraction of the pellet carried out. The supernatants were pooled and to the combined extract saturated NaCl (0.25vol.), water (0.25vol.) and diethyl ether (1vol.) were added. After shaking, the diethyl ether extract (greenish yellow) was removed and the aqueous layer was extracted three times with diethyl ether (1vol.). The combined diethyl ether layers were reduced in volume in a stream of N_2 when a water layer again separated. The dense-green diethyl ether layer was removed by using a Pasteur pipette and evaporated to dryness under a stream of N_2.

Thin-layer chromatography

The residue after esterification of phycocyanobilin was dissolved in chloroform and applied to silica gel G plates (20cm × 20cm × 0.05cm) and developed with chloroform/methyl acetate (2:1, v/v). Two major blue bands were observed, but their proportions varied from one experiment to another. These bands corresponded to those of authentic samples of phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester–methanol adduct (Troxler *et al.*, 1979), which were used as markers. In this system the authentic compounds gave R_F values of 0.73 for phycocyanobilin dimethyl ester and 0.60 for phycocyanobilin dimethyl ester–methanol adduct. The latter compound, which is produced from phycocyanobilin dimethyl ester during methylation and involves the addition of a methanol molecule across the ethylidene side chain, has been described in detail by Beuhler *et al.* (1976). The bands were scraped from the plate, eluted with methanol and evaporated to dryness in a stream of N_2. These purified pigments were used for spectral measurements and scintillation counting.

Chlorophyll *a* was purified by t.l.c. on cellulose plates (20cm × 20cm × 0.1cm) developed with light petroleum (b.p. 40–60°C)/acetone/propan-1-ol (90:10:0.45, by vol.) as described by Bacon (1964). A single green band corresponding to chlorophyll *a* (R_F 0.80) separated from yellow–orange bands corresponding to carotenoids (R_F 0.87 and 0.65). The chlorophyll *a* band was scraped from the plate, eluted in diethyl ether and evaporated to dryness in a stream of N_2.

Radiochemical assay

Samples of bile pigments for counting of radioactivity were dissolved in chloroform and 0.05ml was transferred to a scintillation vial, followed by addition of 5ml of scintillant [0.4% (w/v) 2,5-diphenyloxazole in toluene]. To avoid excessive colour quenching, vials were placed under two 40W fluorescent lights and left overnight to bleach. The resultant solutions (pale purple) were placed in a Beckman LS200B scintillation counter and counted for radioactivity repeatedly over a period of 24h. Whenever possible, samples were counted for 30–50min on two channels; counts were usually constant after approx. 3h in the dark. A quench curve was constructed with chloroform as quenching agent and [^{14}C]hexadecane. C.p.m. values were converted into d.p.m. by using this quench curve and channel ratios. In some cases, counting efficiency was determined by addition of a known number of d.p.m. (with [^{14}C]hexadecane) to sample vials and this gave excellent agreement with the quench-curve method. The final values of d.p.m. calculated were the averages of at least four independent countings. Blank samples (i.e. scintillant only or scintillant containing unlabelled samples) gave approx. 50d.p.m. and this blank was subtracted from all samples. Representative data for radiochemical assay of phycocyanobilin dimethyl ester are shown in Table 1.

Spectra

Electronic spectra were determined on a Unicam SP.1800 spectrophotometer, using stoppered cuvettes.

Results

Determination of molar absorption coefficients

The spectral parameters for phycocyanobilin dimethyl ester in 5% (w/v) HCl in methanol have been determined by Cole *et al.* (1967) as $\lambda_{max.}$ 374 ($\varepsilon = 47.9 \times 10^3$ litre·mol^{-1}·cm^{-1}) and $\lambda_{max.}$ 690nm ($\varepsilon = 37.9 \times 10^3$ litre·mol^{-1}·cm^{-1}). Values for phycocyanobilin dimethyl ester and the methanol adduct have not yet been determined in neutral chloroform, but this information is required when dealing with small quantities of bile pigment, since the same material has to be used both for spectral determination and for counting radioactivity (recovery as a solid is much more difficult from acid methanol than from neutral chloroform). The spectrum of

Table 1. *Determination of radioactivity in phycocyanobilin dimethyl ester*

Phycocyanobilin dimethyl ester was obtained from incubation of cells with 5-amino[4-^{14}C]laevulinate (see Table 3). The total sample was dissolved in 10ml of $CHCl_3$ and 0.5ml portions were assayed for radioactivity as described in the Experimental section. Each portion was counted four times for 30min. Counting efficiencies were determined from a quench curve.

Portion number	Radioactivity found (c.p.m.)	Counting efficiency (%)	Uncorrected radioactivity (d.p.m.)	Radioactivity after blank correction (d.p.m.)	Average (d.p.m.)
1	1461	89.7	1629	1579	1561
	1439		1604	1554	
	1437		1602	1552	
	1445		1611	1561	
2	1431	89.7	1595	1545	1536
	1422		1585	1535	
	1415		1587	1527	
	1424		1588	1538	
3	1561	89.7	1740	1690	1689
	1559		1738	1688	
	1565		1745	1695	
	1556		1735	1685	

Table 2. *Electronic-spectral parameters for phycocyanobilin dimethyl ester*

All samples contained an equal quantity of phycocyanobilin dimethyl ester. The absorption coefficients in acid methanol are those of Cole *et al.* (1967). These were used to determine the corresponding values in neutral chloroform shown in the Table.

Solvent	$\lambda_{max.}$ (nm)	Absorbance	$10^{-3} \times \varepsilon$ (litre·mol^{-1}·cm^{-1})	$\lambda_{max.}$ (nm)	Absorbance	$10^{-3} \times \varepsilon$ (litre·mol^{-1}·cm^{-1})
5% (v/v) HCl in methanol	374	0.340	47.9	690	0.244	37.9
	374	0.344		690	0.247	
Neutral chloroform	369	0.380	54.0	600	0.133	18.9
	369	0.382	54.3	600	0.133	18.9

phycocyanobilin dimethyl ester in neutral chloroform was determined as follows. An appropriate quantity of purified phycocyanobilin dimethyl ester ($R_F = 0.74$) was dissolved in chloroform and 1ml portions of this solution were placed into each of four test tubes. After evaporation to dryness under a stream of N_2, exactly 5ml of chloroform was added to two tubes and 5ml of 5% (v/v) HCl in methanol was added to the other two tubes. Spectra of each were then determined in duplicate. Since each tube contained exactly the same quantity of bile pigment and the absorption coefficients in acid methanol are known, corresponding absorption coefficients in neutral chloroform are readily determined. The results of these determinations are shown in Table 2, from which the average values obtained were $\lambda_{max.} = 369$ ($\varepsilon = 54.2 \times 10^3$ litre·mol^{-1}·cm^{-1}) and $\lambda_{max.} = 600$nm ($\varepsilon = 18.9 \times 10^3$ litre·mol^{-1}·cm^{-1}). The spectrum of phycocyanobilin dimethyl ester in neutral chloroform is shown in Fig. 2.

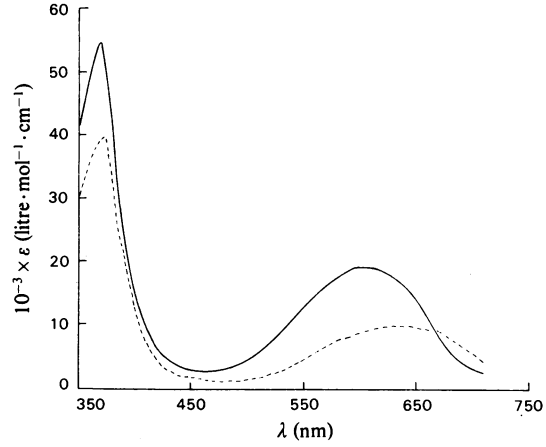

Fig. 2. *Electronic absorption spectra of phycocyanobilin derivatives in neutral chloroform*

——, phycocyanobilin dimethyl ester; – – – –, phycocyanobilin dimethyl ester–methanol adduct.

The corresponding spectral parameters for phycocyanobilin dimethyl ester–methanol adduct in neutral chloroform were determined by a radiochemical method based on the fact that phycocyanobilin dimethyl ester and the methanol adduct of the diester (prepared from the same radiolabelled phycocyanobilin) must necessarily have the same specific radioactivity. Samples of the diester ($R_F = 0.74$) and the methanol adduct ($R_F = 0.57$) prepared from algal cells incubated with 5-amino-[4-^{14}C]laevulinate (without haem) were used for this determination. Specific radioactivities and spectral data are shown in Table 3, from which the values obtained are $\lambda_{max.} = 373$ ($\varepsilon = 39.2 \times 10^3$ litre^{-1}·mol^{-1}·cm^{-1}) and $\lambda_{max.} = 638$ nm ($\varepsilon = 10.3 \times 10^3$ litre^{-1}·mol^{-1}·cm^{-1}). The spectrum of phycocyanobilin dimethyl ester–methanol adduct in neutral chloroform is also shown in Fig. 2.

Uptake of haem by C. caldarium cells

Suspensions of *C. caldarium* incubated at pH 8 showed a marked ability to rapidly remove haem from the suspending medium as shown in Fig. 3. This did not appear to be due to precipitation of haem and the pH of the medium remained constant throughout this incubation. Although it was possible that this haem removal was due to binding at the surface of the cells rather than true uptake and transport into the cell, little or no haem could be removed from the cells by repeated washing with pH 8 buffer or water. It seemed likely therefore that the removed haem had actually been taken up by the algal cells. When such cells were resuspended in acid medium (pH 1.7) it is very unlikely that any haem would be released because of its insolubility at low pH.

^{14}C-labelled haem incorporation into excreted phycocyanobilin

Dark-grown cells of *C. caldarium* were incubated with ^{14}C-labelled haem or unlabelled haem and

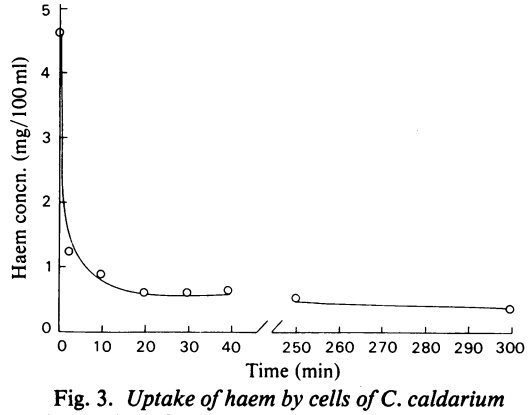

Fig. 3. *Uptake of haem by cells of C. caldarium*
The results refer to an incubation of 4 ml of packed cells in 40 ml of pH 8 medium (see the Experimental section). Samples were withdrawn periodically, the cells were removed by centrifugation and the haem absorbance in the supernatant was measured at 384 nm. The haem concentration in each sample was determined by comparison with the absorbance of a standard haem solution containing no algal cells.

Table 3. *Determination of the molar absorption coefficient for phycocyanobilin dimethyl ester–methanol adduct in neutral chloroform*

Phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester–methanol adduct were obtained from the same experiment in which cells were administered 5-amino[4-^{14}C]laevulinate. It is therefore assumed that each compound has the same specific radioactivity. For phycocyanobilin dimethyl ester, the total sample was dissolved in 10 ml of chloroform and the absorption at 369 nm was determined. Three 0.5 ml portions of this solution were assayed for radioactivity as described in the Experimental section. Thus, from the known molar absorption coefficient at 369 nm (54.21·mol^{-1}·cm^{-1}) the specific radioactivity was found to be 10.05×10^{10} d.p.m./mol. For the methanol adduct of phycocyanobilin dimethyl ester, the total sample was dissolved in 10 ml of chloroform and the absorption spectrum was determined. Two 2 ml portions of this solution were assayed for radioactivity as described in the Experimental section. Thus, from the determined specific radioactivity of phycocyanobilin dimethyl ester, and the measured radioactivity in phycocyanobilin dimethyl ester–methanol adduct (2872 d.p.m.) the molar absorption coefficients for the latter at 373 and 638 nm were calculated.

Compound	$\lambda_{max.}$ (nm)	$10^{-3} \times \varepsilon$ (litre·mol^{-1}·cm^{-1})	Absorbance	Concentration (μM)	Amount counted (nmol)	Radioactivity found (d.p.m.)	$10^{-10} \times$ Specific radioactivity (d.p.m./mol)	$10^{-10} \times$ Average specific radioactivity (d.p.m./mol)
Phycocyanobilin dimethyl ester	369	54.2 (from Table 2)	1.72	31.7	15.87	1561	9.84	10.05
					15.87	1536	9.68	
					15.87	1689	10.64	
Phycocyanobilin dimethyl ester–methanol adduct	373	39.2	0.560	14.3	2.86	2844	2872	
	638	10.3	0.147			2901		

resuspended in the dark in acid medium containing glucose and unlabelled 5-aminolaevulinate as described in the Experimental section. Three experiments were carried out with different proportions of radiolabelled haem and different volumes of culture. In each case the excreted phycocyanobilin was converted into the dimethyl ester and its methanol adduct, which were separately isolated and determined spectroscopically as described above. The pigments obtained from cells incubated with unlabelled haem showed no significant excess counts above background. Bile-pigment samples prepared from cells incubated with ^{14}C-labelled haem showed a significant incorporation of radioactivity as shown in Table 4. Specific activities were determined by using the absorption coefficients found above (Tables 2 and 3).

Effect of unlabelled haem on 5-amino[4-^{14}C]-laevulinate incorporation into phycocyanobilin

The effect of pre-incubation with unlabelled haem on the specific activity of bile pigments produced from 5-amino[4-^{14}C]laevulinate was also examined. Dark-grown cells were resuspended in pH 8 medium with or without unlabelled haem and the cell suspensions were incubated in the dark for 5h. The cells were then resuspended in acid medium as described above and 5-amino[4-^{14}C]laevulinate was added to each. After incubation for 90h in the dark, excreted phycocyanobilin was extracted and purified as described above and specific radioactivities were determined. The results from two groups of experiments, shown in Table 5, clearly show a dilution of the specific radioactivity when the phycocyanobilin is synthesized in the presence of unlabelled haem. These results indicate that unlabelled haem is incorporated into phycocyanobilin.

^{14}C-labelled haem incorporation into phycocyanin

Dark-grown cells, incubated with ^{14}C-labelled haem or unlabelled haem at pH 8 were resuspended in acid medium minus glucose and exposed to light as described above. Samples of phycocyanobilin, cleaved from phycocyanin, were methylated and purified as before. Chlorophyll *a* samples were extracted and purified by t.l.c. as described in the Experimental section. The spectrum of the chlorophyll *a* thereby obtained was determined in diethyl ether and the peak wavelengths showed excellent agreement with literature values (Holt, 1976). The samples were almost free from carotenoids as judged by the lack of absorption peaks around 460nm. The chlorophyll *a* was estimated spectrophotometrically by using the peak at 664nm for which $\varepsilon = 65 \times 10^3$ litre·mol^{-1}·cm^{-1}.

The samples of bile pigment and chlorophyll *a* prepared in the presence of unlabelled haem showed, as expected, no counts above background. The

Table 4. [^{14}C]*Haem incorporation into excreted phycocyanobilin*

The results refer to experiments in which cells were incubated with both 5-aminolaevulinate and [^{14}C]haem as described in the text. In each experiment, phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester–methanol adduct were purified and their specific radioactivities were determined. The fraction of total phycocyanobilin recovered that was derived from added [^{14}C]haem (last column) was calculated as the ratio of the specific radioactivity of phycocyanobilin formed to that of the specific radioactivity of the haem added. Specific radioactivities were determined from absorption spectra by using the data derived in Tables 2 and 3.

Sample	R_F	Culture volume (ml)	Weight of ^{14}C-labelled haem added (mg)	$10^{-10}\times$ Specific radioactivity of added haem (d.p.m./mol)	Total pigment recovered (nmol)	Total radioactivity found (d.p.m.)	$10^{-10}\times$ Specific radioactivity (d.p.m./mol)	Fraction of phycocyanobilin from added haem
Expt. 1								
Phycocyanobilin dimethyl ester	0.72	80	3.8	23.3	88.6	3525	4.0	0.17
Phycocyanobilin dimethyl ester–methanol adduct	0.60				34.1	1395	4.1	0.18
Expt. 2								
Phycocyanobilin dimethyl ester	0.73	40	1.0	23.3	14.6	767	5.2	0.22
Phycocyanobilin dimethyl ester–methanol adduct	0.60				27.7	1291	4.7	0.20
Expt. 3*								
Phycocyanobilin dimethyl ester	0.74	40	1.0	11.7	189	2560	1.4	0.12

* The amount of phycocyanobilin dimethyl ester–methanol adduct was insufficient for radiochemical analysis.

Table 5. *^{14}C Incorporation into phycocyanobilin synthesized from 5-amino[4-^{14}C]laevulinate in the presence and absence of added (unlabelled) haem*

The results refer to experiments in which the effect of added haem on the specific radioactivity of phycocyanobilin excreted by cells administered 5-amino[4-^{14}C]laevulinate was determined. Two groups of experiments were carried out with different proportions of radioactivity added (5-amino[4-^{14}C]laevulinate). Different proportions of haem were used as shown in the Table. The specific radioactivity of phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester–methanol adduct were determined from absorption spectra by using the results given in Tables 2 and 3. For each group of experiments, the control did not contain haem and haem incorporation is seen as a decrease in the specific radioactivity of phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester–methanol adduct, as compared with the control. The fraction of phycocyanobilin formed from added haem was determined by subtracting from unity the ratio of the specific radioactivity of phycocyanobilin obtained in the presence of added haem to that prepared in the absence of added haem.

Sample	Culture volume (ml)	Weight of haem added (mg)	Radioactivity added (μCi)	Total pigment recovered (nmol)	Total radioactivity recovered (d.p.m.)	$10^{-10}\times$ Specific radioactivity (d.p.m./mol)	Fraction of phycocyanobilin from added haem
Phycocyanobilin dimethyl ester	80	0	5.0	317	31900	10.1	0
Phycocyanobilin dimethyl ester–methanol adduct				71.5	7180	10.0	0
Phycocyanobilin dimethyl ester	80	8.0	5.0	231	19800	8.6	0.15
Phycocyanobilin dimethyl ester–methanol adduct				74.3	6163	8.3	0.17
Phycocyanobilin dimethyl ester*	40	0	10.0	132	76300	57.8	0
Phycocyanobilin dimethyl ester*	40	1.0	10.0	141	71700	50.9	0.12
Phycocyanobilin dimethyl ester*	40	3.0	10.0	138	56900	41.2	0.29

* The amount of phycocyanobilin dimethyl ester–methanol adduct was insufficient for radiochemical analysis.

Table 6. *Incorporation of ^{14}C-labelled haem into phycocyanin*

The culture volume was 100ml (10ml of packed cells) and 3.0mg of ^{14}C-labelled haem (specific radioactivity, 23.3×10^{10}d.p.m./mol) was added. The R_F values for bile pigment and chlorophyll *a* refer to the respective systems described in the Experimental section.

Sample	R_F	Total pigment recovered (nmol)	Total radioactivity found (d.p.m.)	$10^{-10}\times$ Specific radioactivity (d.p.m./mol)	Fraction of phycocyanobilin from added haem
Phycocyanobilin dimethyl ester	0.71	50.9	2322	4.6	0.20
Phycocyanobilin dimethyl ester–methanol adduct	0.60	100	4414	4.4	0.19
Chlorophyll *a*	0.80	79.1	0	0	—

results for the experiment using ^{14}C-labelled haem are given in Table 6, which shows incorporation of haem into phycocyanobilin cleaved from phycocyanin, thus demonstrating that administered haem was utilized *in vivo* for synthesis of the chromophore of phycocyanin. It is equally clear that the chlorophyll *a* synthesized concomitantly with phycocyanin contained no radiolabel.

Discussion

Haem uptake by C. caldarium

The present work has demonstrated that, somewhat surprisingly, haem is taken up by algal cells from a pH8 medium. The fact that the added haem is metabolized by cells proves that haem removal from the medium is not simply due to external

binding at the cell surface. The nature of the transport process is completely unknown, but for *C. caldarium* transport of porphyrins, haem and bile pigments seems to be a process that readily occurs, depending on the pH of the outside medium. Haem transport into cells is known to be obligatory for certain organisms for which it is a required growth constituent (Hutner, 1978) and it is possible that the transport of tetrapyrroles into and out of cells is a more general phenomenon than has hitherto been recognized.

Incorporation of haem into phycocyanobilin

These results have demonstrated unequivocally that radioactivity from added haem is incorporated into phycocyanobilin either when the induction of pigment synthesis is achieved in the dark via addition of 5-aminolaevulinate or, in the more natural system, where phycocyanin synthesis occurs in the light. The results certainly could not be explained in terms of radiochemical impurity of the isolated phycocyanobilin (i.e. the counts being due to a small impurity of ^{14}C-labelled haem) because of the purification procedures used for the isolation and because the spectrum of the pigments showed no trace of a Soret band at around 400nm. Because of the relatively high specific radioactivities achieved, any such impurity would need to be so extensive that a very large Soret peak would be observed.

The possibility that the added haem may lose its iron atom, thereby labelling the protoporphyrin IX pool, needs to be considered. Although such a reaction may be considered unlikely, it could occur in principle by the reversal of the ferrochelatase reaction or possibly non-enzymically in the acid medium. It was primarily to examine this possibility that chlorophyll *a* was isolated from pigmented algal cells that had been incubated in the light. If protoporphyrin IX was labelled then chlorophyll *a* (for which it is a precursor; see Fig. 1) should also be labelled. The lack of label found in chlorophyll *a* indicates that the protoporphyrin IX pool was not labelled and that haem does not lose its iron atom in the system studied. It may therefore be concluded that haem is a precursor on the biosynthetic pathway to phycocyanobilin and the magnesium-mediated pathway may therefore be excluded.

There was some variability in our experiments, both in the final yield of pigment and in the degree of haem incorporation achieved (see e.g. Table 5) and this was probably due to slight variations in the metabolic states of the algal cells after growth. Nevertheless, in general the specific radioactivities of the phycocyanobilin derivatives achieved in the ^{14}C-labelled haem experiments are of the order of 15–20% that of the haem used. This indicates (as shown in Tables 4 and 6) that, of the phycocyanobilin formed, about 15–20% is derived from haem and the rest from added 5-aminolaevulinate (dark experiments) or from endogenous substrates (light experiments). Although they show more variability the haem dilution experiments with 5-amino[4-^{14}C]laevulinate are consistent with this, giving between 15 and 29% dilution (Table 5). This may be regarded as a reasonably good incorporation for a molecule of the size of haem with possible difficulties in intracellular transport.

General discussion

The present results show that haem is a precursor of the phycocyanobilin of *C. caldarium* and that a degradation reaction analogous to that catalysed by haem oxygenase must occur. However, side-chain modification is essential and some or all of this may occur before macrocyclic ring cleavage. Thus it is possible that protohaem could be modified perhaps to mesohaem and that a haem oxygenase with a substrate specificity different to that of the mammalian enzyme may be involved. This may explain why attempts to demonstrate haem oxygenase in algae by using protohaem as substrate have not yet succeeded. The potential for the complete elucidation of the pathway now exists, since various labelled substrates (such as mesohaem) may be administered to algal cells and the incorporation monitored. Although the generally accepted structures of the biliprotein chromophores *in vivo* have recently been questioned (O'Carra *et al.*, 1980; Killilea *et al.*, 1980), the results presented here for the origin of phycocyanobilin are clearly independent of such discussions.

Although the present work directly refers only to the phycocyanobilin of *C. caldarium*, it seems very likely that the results achieved may be generalized to all plant bile pigments including phytochrome.

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