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Phycocyanobilin Synthesis in the Unicellular Rhodophyte *Cyanidium caldarium*

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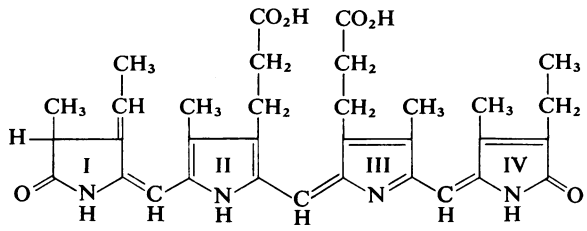
Light is required for synthesis of the accessory photosynthetic pigment phycocyanin in cells of the unicellular rhodophyte *Cyanidium caldarium*. Phycocyanin is a conjugated protein composed of polypeptide subunits to which the light-absorbing bile pigment chromophore phycocyanobilin is covalently attached. Dark-grown cells of *C. caldarium* are unable to make phycocyanin, but when incubated in the dark with 5-aminolaevulinate the cells synthesize and excrete a protein-free phycobilin (algal bile pigment) into the suspending medium. The electronic absorption spectrum, electron impact mass spectrum, chromatographic properties and imide products obtained after chromic acid degradation of the excreted phycobilin were identical with those of phycocyanobilin cleaved from phycocyanin in boiling methanol. This establishes the structural identity between the excreted phycobilin, which is the end product of bile-pigment synthesis *in vivo*, and the chromophore cleaved from phycocyanin in boiling methanol. The significance of the structure of the excreted phycobilin with respect to the events surrounding the assembly of the phycocyanin molecule *in vivo* is discussed.

Phycobiliproteins are accessory pigments found in the photosynthetic apparatus of red (Rhodophyta), blue–green (Cyanophyta) and cryptomonad (Cryptophyta) algae (O'Carra & Ó hEocha, 1976). Allophycocyanin, phycocyanin and phycoerythrin are the principal phycobiliproteins in these organisms (Bogorad, 1975). Phycocyanobilin is the light-absorbing chromophore of allophycocyanin and phycocyanin, and phycoerythrobilin is the major chromophore of phycoerythrin (Bennett & Siegelman, 1977). Phycocyanobilin and phycoerythrobilin are linear tetrapyrroles structurally related to mammalian bile pigments (Siegelman *et al.*, 1968; Troxler, 1977). Algal bile pigments (phycobilins) are synthesized from 5-aminolaevulinate via the porphyrin-synthetic pathway and ultimately arise from the carbon skeleton of protoporphyrin IX, i.e. haem (Troxler, 1972). Phycocyanobilin is derived from its porphyrin precursor by a mechanism (Troxler & Brown, 1977) identical with that used for haem conversion into bile pigment in mammals (Brown & King, 1975).

Phycobilins are covalently linked to apoproteins in algal cells, but the exact nature of the phycobilin–apoprotein linkages has not been definitively established (Chapman, 1973; O'Carra & Ó hEocha, 1976). The classic studies by Lemberg (1928, 1930) demonstrated that algal bile pigments were released from phycobiliproteins in hot concentrated acid and alkali, but, recognizing that the hydrolytic conditions yielded bile-pigment artifacts, Lemberg (1928, 1930) referred to the native chromophores of phycocyanin and phycoerythrin as phycocyanobilin and phycoerythrobilin respectively. More recently, phycobilins have been cleaved from apoproteins by hydrolysis in concentrated acid at room temperature (Ó hEocha, 1963; Rudiger & O'Carra, 1969) or by boiling phycobiliproteins in methanol (Fujita & Hattori, 1963; Cole *et al.*, 1968; Siegelman *et al.*, 1968; Troxler, 1972). Ó hEocha (1963) showed that the phycobilin obtained by hydrolysis of phycocyanin in concentrated HCl at room temperature was spectrally identical with the covalently attached chromophore of phycocyanin in 8M-urea, and he regarded the cleaved phycobilin as phycocyanobilin. This phycobilin has not been crystallized and its structure is not known (O'Carra & Ó hEocha, 1976). The phycobilin isolated from phycocyanin by boiling methanol has been purified as the dimethyl ester derivative, and its structure has been determined by degradative tech-



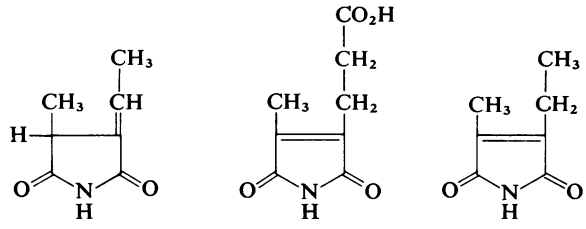


Fig. 1. *Structures of phycocyanobilin and imides derived from phycocyanobilin*
The imides were obtained by degradation of phycocyanobilin in 1% (w/v) CrO_3/1 M-H_2SO_4 at 100°C for 30min (see the text).

niques (Rudiger, 1968; Rudiger & O'Carra, 1969), mass spectrometry and n.m.r. spectroscopy (Cole *et al.*, 1968; Crespi *et al.*, 1968; Schram & Kroes, 1971; Beuhler *et al.*, 1976). This phycobilin has been considered in some laboratories to be synonymous with phycocyanobilin (Fig. 1) (Siegelman *et al.*, 1968; Troxler, 1972; Beuhler *et al.*, 1976) and is referred to as such below.

Troxler & Bogorad (1966) found that cells of the unicellular rhodophyte *Cyanidium caldarium* excreted a protein-free phycobilin into the growth medium when incubated with 5-aminolaevulinate, the first committed intermediate in the porphyrin pathway. The excreted phycobilin was considered to be the linear tetrapyrrole (i.e. bile pigment), which becomes covalently attached to apoprotein during phycocyanin biosynthesis *in vivo* (Troxler & Lester, 1967; Troxler, 1972).

In the present paper, the properties of the excreted phycobilin are compared with those of phycocyanobilin cleaved from phycocyanin by boiling methanol, and the relationship between the excreted phycobilin and phycocyanin biosynthesis *in vivo* is discussed.

Experimental

Organism

Cyanidium caldarium (Allen, 1959) is a unicellular rhodophyte that synthesizes chlorophyll *a*, allophycocyanin and phycocyanin in the light, but is unable to make these photosynthetic pigments when grown in the dark. Dark-grown cells synthesize photosynthetic pigments, however, when placed in the light. *C. caldarium* mutant III-D-2 was used in the present investigation because this strain produces more pigment per cell in the light than does the wild-type (Troxler & Bogorad, 1966).

Culture conditions

Algal cells were grown from a small inoculum on a rotary shaker in 1500ml of medium supplemented with 1% (w/v) glucose (Allen, 1959) in 3-litre Fernbach flasks at 37°C in the dark for 7–10 days (standard conditions). Dark-grown cells were subsequently treated in one of two ways. (*a*) Dark-grown algal cells were collected by centrifugation (15000*g* for 15min), resuspended in medium (1500ml) minus glucose and 5-aminolaevulinate, and incubated for 72h under standard conditions in the light (General Electric fluorescent tubes, F17PG, CW; approx. 5400lx). The cells produced chlorophyll *a* and phycobiliproteins in the light. Phycocyanin was isolated and purified from these pigmented cells as described below. (*b*) Algal cells were collected by centrifugation at 15000*g* for 5min, and 1ml of packed cells was placed in 9ml of medium containing 1% glucose and 50mM-5-aminolaevulinate (Sigma Chemical Co., St. Louis, MO, U.S.A.), and several such suspensions were incubated in 25ml Erlenmeyer flasks for 72h in the dark. The phycobilin synthesized from 5-aminolaevulinate under these conditions was isolated and purified as described below.

Preparation of phycocyanin

After illumination, algal cells were collected by centrifugation at 15000*g* for 5min, resuspended in 0.1M-phosphate buffer (KH_2PO_4/K_2HPO_4), pH7.0 (1ml of cells/10ml of buffer), and disrupted by sonication with a Branson sonic oscillator (model W 185) for 10min as described by Brown & Troxler (1977). Particulate matter was removed by centrifugation at 36000*g* for 30min and the resulting supernatant was made 50% saturated with $(NH_4)_2SO_4$. The precipitated phycocyanin (and small quantities of allophycocyanin) were collected by centrifugation, dialysed overnight against water (50vol.) at 4°C and the dialysed samples were centrifuged at 100000*g* for 1h to remove traces of chlorophyll *a*. Phycocyanin in the supernatant was purified by ion-exchange chromatography on brushite columns (2.5cm × 20cm) developed with potasium phosphate buffers (pH7.0) of increasing ionic strength as described by Brown & Troxler (1977). Phycocyanin, which was eluted from the columns in 0.01–0.025M-potassium phosphate buffer, was precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation, and the precipitate was dialysed overnight against water (50 vol.) at 4°C and freeze-dried.

Preparation of phycocyanobilin

Phycocyanin (100mg) was suspended in 50ml of 1% (w/v) trichloroacetic acid and incubated with occasional stirring at room temperature (25°C) for 3h. The denatured phycocyanin was collected by centrifugation (40000*g* for 30min), and washed with 3 × 20ml of water, 3 × 20ml of methanol and 3 × 20ml of diethyl ether. The washed sample was suspended in 500ml of methanol and refluxed for 16h. The solution was cooled, filtered through Whatman no. 1 filter paper to remove denatured protein, and the filtrate containing phycocyanobilin (free acid) was evaporated to dryness under reduced pressure at 50°C.

Isolation of excreted phycobilin

Algal cells that had been incubated with 5-aminolaevulinate in the dark for 72h were collected by centrifugation (15000*g* for 15min), and the phycobilin in the supernatant (growth medium supplemented with glucose and 5-aminolaevulinate 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.

Esterification

The phycobilin synthesized from 5-aminolaevulinate in the dark and phycocyanobilin isolated from phycocyanin in boiling methanol were each dissolved in 5ml of methanol; 5ml of 14% (w/v) BF_3 in methanol (Applied Science Laboratories, State College, PA, U.S.A.) was added and the solution was refluxed for 2min. The solution was cooled to room temperature, 50ml of water was added and the algal bile-pigment diesters were extracted into chloroform. The chloroform solution was washed with water, filtered through chloroform-moistened filter paper, evaporated to dryness under a stream of N_2 and the product stored at −20°C until used.

Thin-layer chromatography

The bile-pigment dimethyl esters were dissolved in chloroform, applied to 20cm × 20cm silica-gel plates (Absorbosil-5; Applied Science Laboratories) and chromatographed in one of the following solvent systems (Cole *et al.*, 1968): (1) methylene dichloride/ethyl acetate (4:1, v/v); (2) benzene/ethanol (9:1, v/v); (3) carbon tetrachloride/methyl acetate (2:1, v/v). The band of purified bile-pigment diester was scraped off the chromatogram, eluted from silica gel with chloroform or ethanol, which was evaporated to dryness, and the methylated bile pigments were stored at ~20°C until used.

Chromic acid degradation

Algal bile pigments (free acids or dimethyl esters) were dissolved in 1% CrO_3/1M-H_2SO_4 (1ml) and incubated at 100°C for 30min in a Thermoblock (LaPine Instruments, Melrose Park, IL, U.S.A.). The cooled samples were extracted with 3 × 1ml of ethyl acetate and the combined extracts were evaporated to dryness under N_2. The residues were dissolved in ethyl acetate, applied to 20cm × 20cm silica-gel plates (250μm thick; Redi-Plate; Fisher Scientific, Pittsburgh, PA, U.S.A.) and chromatographed in carbon tetrachloride/ethyl acetate/cyclohexane (6:3:1, by vol.). Imides derived from algal bile pigments were detected by spraying with chlorine/benzidine in a fume cupboard (Rudiger, 1968). Rudiger & O'Carra (1969) have shown that chromic acid degradation of phycocyanobilin at 100°C yields ethylidinemethylsuccinimide from ring I, haematinic acid from rings II and III, and ethylmethylmaleimide from ring IV (Fig. 1). Authentic imide standards of ethylidinemethylsuccinimide, haematinic acid and ethylmethylmaleimide were kindly provided by Dr. Hans-Peter Köst, Botanische Institut, Universität der München, München, Germany.

Mass spectrometry

Mass spectra of algal bile pigments were determined on an A.E.I. MS9 mass spectrometer.

Results

Phycobilin synthesis

Dark-grown *C. caldarium* cells excreted a protein-free phycobilin into the suspending medium when

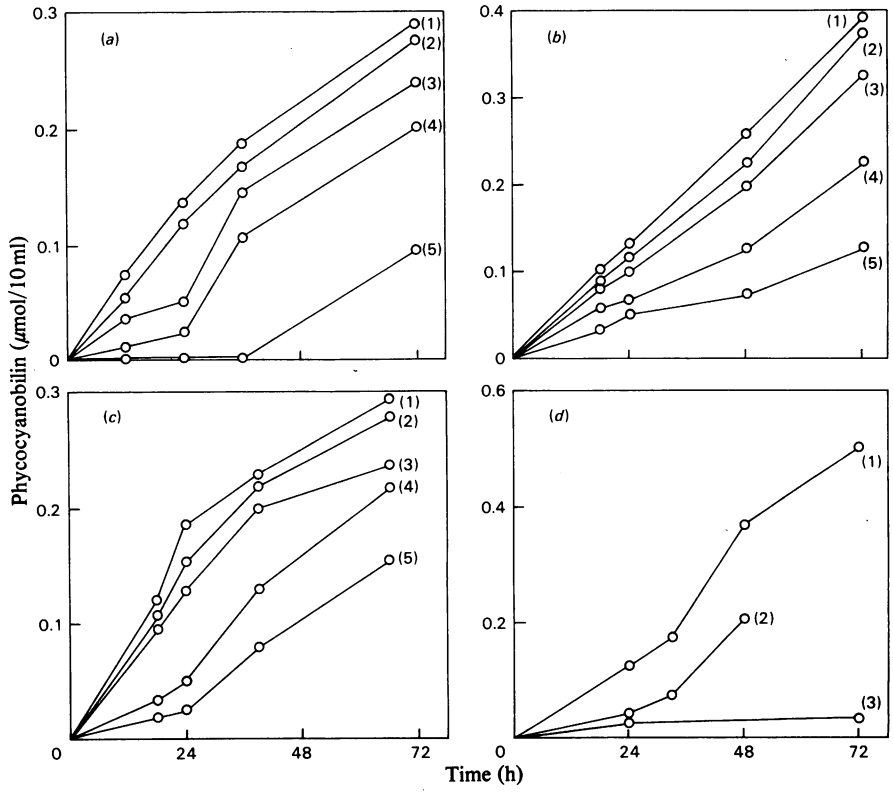


Fig. 2. *Effects of 5-aminolaevulinate concentration, glucose concentration, amount of packed cells added and inhibitors of protein synthesis on the kinetics of phycobilin excretion from C. caldarium cells*

At intervals, 1–2ml of the incubation mixture was removed, the cells were collected by centrifugation (15000*g* for 15min) and discarded, and the supernatant growth medium was extracted with 3 × 1ml of chloroform. The quantity of excreted phycobilin in the pooled chloroform extracts was estimated spectrophotometrically (ε_{mM} = 38 at 658nm in acid/chloroform; Bennett & Siegelman, 1977). Except for the parameter being varied, incubations were performed under standard conditions with 1ml of packed cells added to 9ml of growth medium supplemented with 1% (w/v) glucose and 50mM-5-aminolaevulinate. (*a*) Variation of 5-aminolaevulinate concentration: (1) 75mM; (2) 50mM; (3) 25mM; (4) 10mM; (5) 75mM with glucose not initially present and added after 36h. (*b*) Variation of glucose concentration: (1) 0.3M; (2) 0.15M; (3) 70mM; (4) 35mM; (5) 15mM. (*c*) Variation of amount of packed cells added: (1) 2.0ml; (2) 1.0ml; (3) 0.5ml; (4) 0.25ml; (5) 0.12ml. (*d*) Effect of inhibitors of protein synthesis: (1) control; (2) addition of chloramphenicol to final concentration of 3mM; (3) addition of cycloheximide to final concentration of 50μM.

incubated in medium supplemented with 1% glucose and 50mM-5-aminolaevulinate (Fig. 2). This is an unusual process because phycocyanin (and its phycocyanobilin chromophore) synthesis normally requires light and all of the chromophore produced becomes covalently attached to apoprotein (Troxler, 1972). The results in Fig. 2 show that phycobilin synthesis from 5-aminolaevulinate in the dark requires a reduced carbon source and is dependent on the concentration of glucose, 5-aminolaevulinate and amount of packed cells added. Phycobilin synthesis and excretion were inhibited almost totally by cycloheximide, but chloramphenicol caused only a lag in such synthesis and excretion.

Phycobilin spectral properties

Algal cells incubated under the standard conditions described above were collected by centrifugation (15000*g* for 15min) and discarded. The (excreted) phycobilin free acid in the resulting supernatant (suspending medium) was isolated as described above and was examined spectrophotometrically in several solvents (Table 1). The phycobilin displayed a broad absorption band from 600 to 615nm in neutral

chloroform. This band was red-shifted to 658nm and 680nm in acid chloroform and 5% (v/v) HCl in methanol respectively, indicating that a pyrrolenine (–N=) nitrogen atom on one of the pyrrole rings was present on the phycobilin at neutral pH (Ó hEocha, 1963).

The phycobilin free acid was converted into the dimethyl ester derivative and purified by t.l.c. The absorption maxima and chromatographic properties of the phycobilin dimethyl ester are compared with those of phycocyanobilin dimethyl ester (free acid cleaved from phycocyanin in boiling methanol and esterified) in Tables 2 and 3. The excreted and cleaved bile-pigment diesters were spectrally indistinguishable as the free base [in 5% (v/v) pyridine in chloroform or methanol] and as the hydrochloride [in 5% (v/v) HCl in methanol]. Similarly, the R_F values of the excreted and cleaved bile-pigment diesters were the same on silica gel in three solvent systems.

Chromic acid degradation

The patterns of imides derived from the free acid and dimethyl ester derivative of the excreted phycobilin and that of phycocyanobilin cleaved from phycocyanin are indicated diagrammatically in Fig. 3. Also shown is the pattern for mesobiliverdin pre-

Table 1. *Spectral properties of the phycobilin free acid excreted by C. caldarium cells incubated with 50mM-5-aminolaevulinate in the dark for 72h*

A sample (10μl) of bile pigment free acid in chloroform was added to 3ml of the solvents indicated and spectra were determined on a Beckman DBGT recording spectrophotometer. λ_1 and λ_2 represent the wavelengths of the long- and short-wavelength maxima respectively, and $\varepsilon_{\lambda 1}$ and $\varepsilon_{\lambda 2}$ represent the appropriate molar absorption coefficients.

Solvent	λ_1 (nm)	λ_2 (nm)	$\varepsilon_{\lambda 2}/\varepsilon_{\lambda 1}$
Neutral chloroform	600–615	370	2.8
Acid chloroform	658	367	1.6
[3M-HCl/chloroform (1:3, v/v)]			
5% (v/v) HCl in methanol	680	372	1.1

Table 3. *Chromatographic properties of the excreted phycobilin (dimethyl ester derivative) and phycocyanobilin (dimethyl ester derivative) cleaved from C. caldarium phycocyanin*

Chromatography was carried out on silica-gel plates (see the Experimental section). Solvent 1, methylene dichloride/ethyl acetate (4:1, v/v); solvent 2, benzene/ethanol (9:1, v/v); solvent 3, carbon tetrachloride/methyl acetate (2:1, v/v).

Bile-pigment diester	R_F		
	Solvent 1	Solvent 2	Solvent 3
Phycobilin (excreted)	0.29	0.63	0.43
Phycocyanobilin (cleaved)	0.29	0.66	0.44

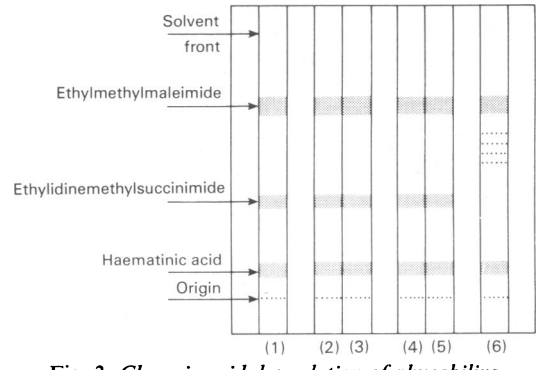


Fig. 3. *Chromic acid degradation of phycobilins*

T.l.c. was carried out as described in the text. (1) Imide standards; (2) excreted phycobilin free acid; (3) excreted phycobilin dimethyl ester; (4) phycocyanobilin (cleaved) free acid; (5) phycocyanobilin dimethyl ester (cleaved); (6) mesobiliverdin dimethyl ester.

Table 2. *Spectral properties of the dimethyl ester of the phycobilin excreted as the free acid by C. caldarium cells incubated with 50mM-5-aminolaevulinate in the dark for 72h, and of the dimethyl ester of phycocyanobilin cleaved as the free acid from phycocyanin in boiling methanol*

A sample (10–50μl) of bile pigment diester in chloroform was added to 3ml of the solvents indicated and the spectrum was determined on a Beckman DBGT recording spectrophotometer. λ_1 and λ_2 represent the wavelengths the long- and short-wavelength maxima respectively, and $\varepsilon_{\lambda 1}$ and $\varepsilon_{\lambda 2}$ represent the appropriate molar absorption coefficients.

	5% (v/v) pyridine in chloroform			Ethanol			5% (v/v) HCl in methanol		
Bile pigment diester	λ_1 (nm)	λ_2 (nm)	$\varepsilon_{\lambda 2}/\varepsilon_{\lambda 1}$	λ_1 (nm)	λ_2 (nm)	$\varepsilon_{\lambda 2}/\varepsilon_{\lambda 1}$	λ_1 (nm)	λ_2 (nm)	$\varepsilon_{\lambda 2}/\varepsilon_{\lambda 1}$
Phycobilin (excreted)	599	367	2.8	605	362	2.9	685	374	1.3
Phycocyanobilin (cleaved)	599	367	2.8	605	362	3.0	685	374	1.3

pared from phycocyanobilin by isomerization in 10% (w/v) KOH in methanol (Rudiger & O'Carra, 1969). The presence of ethylidinemethylsuccinimide in the chromic acid digest of the free acid and dimethyl ester derivative of the phycobilin excreted by cells incubated with 5-aminolaevulinate establishes that ring I (Fig. 1) was reduced and contained an ethylidine substituent. The presence of haematinic acid and ethylmethylmaleimide establishes that the excreted phycobilin was otherwise structurally identical with phycocyanobilin. This methodology was used initially by Rudiger (1968) and Rudiger & O'Carra (1969) to propose the structure of phycocyanobilin.

It should be mentioned that the esterified carboxyl groups of the excreted and cleaved bile pigments (diesters) were hydrolysed during chromic acid degradation in 1% CrO_3 (w/v)/1M-H_2SO_4 at 100°C for 30min. Therefore rings II and III were recovered as haematinic acid rather than haematinic acid methyl ester. Ethylidinemethylsuccinimide was not observed in the chromic acid digest of mesobiliverdin, because ring I of this bile pigment is not reduced and is recovered as ethylmethylmaleimide. The absence of ethylidinemethylsuccinimide from the digest of mesobiliverdin distinguishes this compound from the excreted phycobilin.

Mass-spectral analyses

The electron-impact mass spectra of the excreted phycobilin dimethyl ester and phycocyanobilin dimethyl ester are indicated in Fig. 4. Both bile-

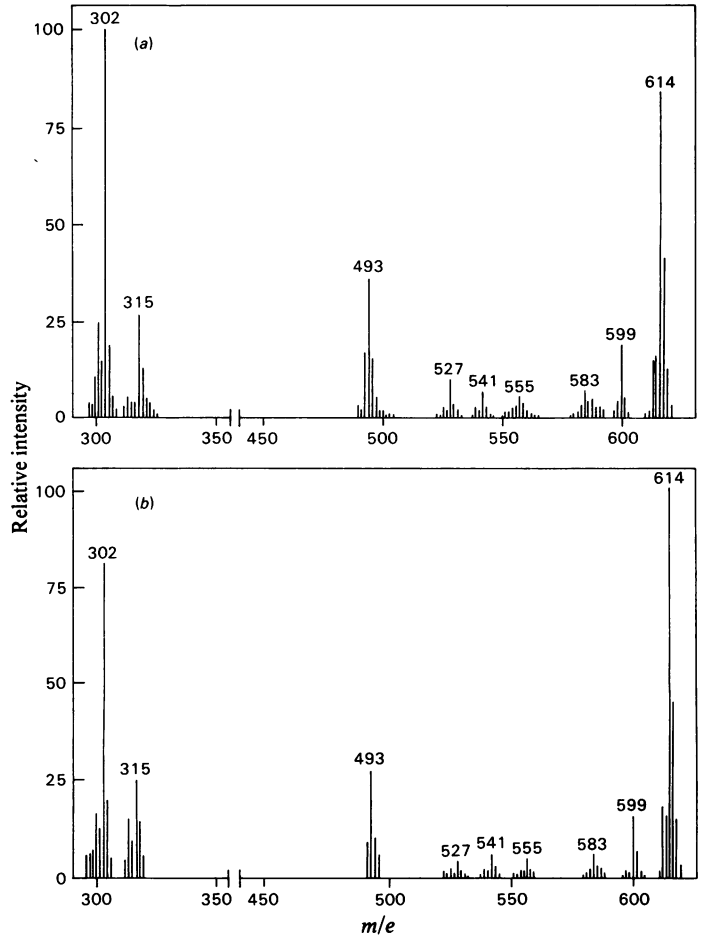


Fig. 4. *Electron-impact mass spectra of phycobilins*
(*a*) Excreted phycobilin dimethyl ester; (*b*) phycocyanobilin dimethyl ester (cleaved). The mass spectra were recorded at 220°C.

pigment diesters displayed major ions at *m/e* 614, 599, 493 and 302, with minor ions at *m/e* 583, 555, 541 and 527. Within any reasonable criteria of mass spectrometry, these two compounds were identical. The spectra in Fig. 4 are in good agreement with the mass spectrum of phycocyanobilin dimethyl ester reported by Cole *et al.* (1968), Schram & Kroes (1971) and Beuhler *et al.* (1976).

The mass spectrum of phycocyanobilin dimethyl ester in Fig. 4 deserves further comment. Beuhler *et al.* (1976) noted that, under controlled conditions of sample evaporation from a Teflon probe at ionization-chamber temperatures below 200°C, there was a large peak at *m/e* 599 in the mass spectrum of phycocyanobilin dimethyl ester. In contrast, the isomeric mesobiliverdin dimethyl ester displayed a small peak at *m/e* 599, and it appeared from these results that this peak might be used to distinguish phycocyanobilin dimethyl ester and mesobiliverdin dimethyl ester in the mass spectrometer.

We were unable to obtain the mass spectrum of phycocyanobilin dimethyl ester at temperatures below 200°C. However, as the temperature was raised from 200 to 250°C the intensity of the peak at *m/e* 599 in the mass spectrum of phycocyanobilin dimethyl ester decreased sharply. Concomitantly, the peak at *m/e* 302 increased relative to that at *m/e* 614. Thus the peak at *m/e* 599 in the mass spectrum of phycocyanobilin dimethyl ester displayed a pronounced temperature effect and as such does not appear to be a good criterion for distinguishing between the dimethyl esters of phycocyanobilin and mesobiliverdin.

Discussion

The present investigation has shown that the electronic absorption spectra, the electron-impact mass spectra, the chromatographic properties and the imide-oxidation products of the phycobilin excreted by *C. caldarium* cells incubated with 5-aminolaevulinate are identical with those of phycocyanobilin cleaved from phycocyanin in boiling methanol. This strongly suggests that the structure of phycocyanobilin shown in Fig. 1 is that of the native chromophore and is not an artifact consequent on cleavage in boiling methanol. It also has interesting implications with respect to the events surrounding assembly of the phycocyanin molecule *in vivo*.

The direct precursor of algal bile pigment has not been unequivocally demonstrated, but both magnesium protoporphyrin IX and iron protoporphyrin IX (haem) have been suggested as possible compounds (Bogorad & Troxler, 1967; Bogorad, 1975; O'Carra, 1975). In algae, the carbon skeleton of a porphyrin molecule is cleaved to one molecule of CO and one molecule of phycocyanobilin (Troxler, 1972; Troxler & Dokos, 1973). There is also a 1:1 stoicheiometry between CO and biliverdin produced during physiological haemoglobin turnover in man and laboratory animals (Sjostrand, 1952; Landau *et al.*, 1970). Brown & King (1976) have shown by measuring the incorporation of isotopically labelled O_2 into mammalian bile pigment *in vivo* and *in vitro* that the oxygen atoms on the outer rings (I and IV) arise from different O_2 molecules (Two-Molecule Mechanism). Troxler & Brown (1977) found that the pattern of $^{18,18}O_2$ incorporation into phycocyanobilin in *C. caldarium* was also consistent with the Two-Molecule Mechanism. This provided evidence for a comparable mechanism of bile-pigment synthesis in algae and mammals and suggests that phycocyanobilin synthesized from 5-aminolaevulinate (via haem) in *C. caldarium* arises from an, as yet undescribed, 'algal haem oxygenase'.

As a working hypothesis, the overall synthesis of the prosthetic group of phycocyanin can be visualized as occurring in three stages: (1) haem cleavage to bile pigment (presumably biliverdin), (2) reduction of the vinyl side chains of biliverdin and isomerization of the resulting mesobiliverdin to phycocyanobilin with the introduction of an ethylidine group on ring I (it is also possible that reduction of vinyl side chains occurs before porphyrin-ring cleavage), and (3) covalent attachment of phycocyanobilin to apoprotein. The results of the present investigation show that stages 1 and 2 precede stage 3.

Finally, several conclusions can be drawn from the effects of incubation conditions on synthesis of phycocyanobilin from 5-aminolaevulinate (Fig. 2). The observed formation of phycocyanobilin from 5-aminolaevulinate indicates that synthesis of 5-aminolaevulinate and its precursors occurs normally during phycocyanin formation in the light. Because of the longer incubation period required for phycocyanobilin synthesis and excretion in the dark than in the light, it is uncertain whether the enzymes that convert the porphyrin ring into phycocyanobilin are constitutive or are induced after the cells have been exposed to added 5-aminolaevulinate. In addition, phycocyanin apoprotein, which cannot be detected in dark-grown *C. caldarium* cells (Seng, 1976), may play a role in the regulation of the porphyrin pathway (haem and phycocyanobilin synthesis) during phycocyanin formation in the light. Since phycocyanobilin synthesis does not require light, the possibility of a photochemical mechanism for algal bile-pigment formation comparable with the photochemical conversion of magnesium protoporphyrin dimethyl ester into protobiliverdin dimethyl ester (Barrett, 1967) seems unlikely. The duration of the incubation is sufficiently long so that it is not surprising that respiratory energy production (from glucose) is required for cell maintenance. Cycloheximide inhibition of phycocyanobilin synthesis

shows that protein synthesis is required for phycocyanobilin synthesis from 5-aminolaevulinate in the dark. This could involve 5-aminolaevulinate induction of the enzymes needed to convert haem into phycocyanobilin, but the exact step(s) at which protein synthesis is required remain(s) to be elucidated.

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