Biosynthesis of the chromophore of phycobiliproteins

A study of mesohaem and mesobiliverdin as possible intermediates and further evidence for an algal haem oxygenase

Stanley B. BROWN and J. Andrew HOLROYD

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

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The possible roles of mesohaem and mesobiliverdin as metabolic precursors of phycocyanobilin, the chromophore of phycocyanin, were studied in the unicellular rhodophyte *Cyanidium caldarium*. Dark-grown cells of this organism, which had been exposed to mesohaem, were either incubated in the dark with 5-aminolaevulinate, which results in excretion of bilins into the suspending medium, or incubated in the light, which results in synthesis of phycocyanin within the cells. By using ^{14}C -labelling, either in the mesohaem or in the 5-aminolaevulinate administered, it was shown that mesohaem is not a precursor of phycocyanobilin in either dark or light systems. However, mesohaem was converted into mesobiliverdin in both systems, a phenomenon that is further evidence for the existence of an algal haem oxygenase. The data also showed that mesobiliverdin is not a precursor of phycocyanobilin. These results suggest that algal bilins are formed via haem degradation to biliverdin in the same way as mammalian bile pigments.

Phycobiliproteins are photoactive pigments that play important roles in photosynthesis (e.g. phycocyanin and phycoerythrin in algae) and in photomorphogenesis (phytochrome in higher plants). Several recent studies have established that the biosynthesis of phycocyanobilin (Fig. 1), the active chromophore of phycocyanin, proceeds via the formation and subsequent degradation of haem (Troxler *et al.*, 1979; Brown *et al.*, 1980, 1981, 1982). These results imply the presence of an algal and plant haem oxygenase carrying out a function analogous to that of the well-established mammalian enzyme (EC 1.14.99.3), which catalyses macrocyclic ring cleavage of haem to biliverdin and thence to bilirubin. However, the presence of this enzyme in plants has not yet been directly demonstrated.

The biosynthesis of phycocyanobilin requires not only haem cleavage to a linear tetrapyrrole, but also two reduction steps. By comparison with mammalian bile-pigment formation, a likely pathway for phycocyanobilin synthesis would be haem cleavage to biliverdin, followed by two reduction steps. However, it is possible that one or both of these reduction steps could take place before macrocyclic ring cleavage and that the true substrate for the algal haem oxygenase may be a reduced haem species. One such compound is

mesohaem (Fig. 1), in which both the vinyl groups of protohaem have been reduced to ethyl groups. Macrocyclic ring cleavage of mesohaem would yield mesobiliverdin (Fig. 1), which is a structural isomer of phycocyanobilin. The final step in phycocyanobilin synthesis would then be an isomerization to form an ethylidene group (Fig. 1). Alternatively, mesobiliverdin could be an intermediate even if mesohaem were not, since it could be formed by reduction of biliverdin, produced by macrocyclic cleavage of protohaem.

In the present work we have studied the metabolism of mesohaem and mesobiliverdin in the unicellular rhodophyte *Cyanidium caldarium*, which synthesizes phycocyanin and chlorophyll *a* when grown photosynthetically. This organism may also be cultured in the dark, when it grows aerobically without photosynthetic pigments. However, such dark-grown cells may be induced to synthesize tetrapyrrolic pigments in two ways. First, exposure to light results in synthesis of phycocyanin and chlorophyll *a*. Alternatively, when incubated with 5-aminolaevulinate and glucose in the dark, such cells do not synthesize significant amounts of phycocyanin, but excrete its chromophore, phycocyanobilin, and also porphyrins into the suspending medium. The detailed use of *C. caldarium* in biosynthetic studies relating to

Fig. 1. Possible role of mesohaem and mesobiliverdin in phycocyanobilin synthesis

Abbreviations used: M, $-\text{CH}_3$; V, $-\text{CH}=\text{CH}_2$; P, $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$; E, $-\text{CH}_2\text{CH}_3$; A, $-\text{CH}=\text{CH}_3$.

algal bile pigments has been described previously (Brown *et al.*, 1981).

Experimental

Materials

All materials obtained commercially were of analytical grade where possible. Solvents were redistilled before use. 5-Amino[4- ^{14}C]laevulinic acid hydrochloride (sp. radioactivity approx. 40 Ci/mol) was obtained from Amersham International, Amersham, Bucks., U.K. ^{14}C -labelled haem (protohaem) was prepared by incubating reticulocyte-rich rabbit blood with 5-amino[4- ^{14}C]laevulinic acid as described previously (Brown *et al.*, 1981). The radiochemical purity of this ^{14}C -labelled haem was confirmed by the method of Bissell *et al.* (1979). This procedure demonstrated that ^{14}C -labelled haem was not contaminated with 5-amino[4- ^{14}C]laevulinate.

Mesohaem (both unlabelled and ^{14}C -labelled) was obtained by catalytic hydrogenation of protohaem on palladinized charcoal (Falk, 1964; Hatzikonstantinou & Brown, 1979). The specific radioactivity of ^{14}C -labelled protohaem was measured as described previously (Brown *et al.*, 1981). In this method haem is determined as the pyridine haemochrome. The specific radioactivity of ^{14}C -labelled mesohaem was determined in a similar way, the absorbance of the pyridine mesohaemochrome being read at 547 nm (Falk, 1964).

Cells of *C. caldarium* (mutant III-D-2) were cultured as described previously (Allen, 1959; Brown *et al.*, 1981).

Incubation of *C. caldarium* with mesohaem in the dark

Dark-grown cells were harvested from acid medium and resuspended in medium at pH 8 (1 ml of packed cells/10 ml of medium) as described previously (Brown *et al.*, 1981). Concentrated mesohaem solutions were prepared by dissolving a weighed quantity of the mesohaem in a minimum volume (less than 0.4 ml) of 0.1 M-NaOH. The 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. This procedure was adopted to permit the cells to take up the mesohaem in the same manner as that described previously for the uptake of protohaem (Brown *et al.*, 1981). After approx. 4 h, the cells were harvested by centrifugation (bench centrifuge), washed twice with water and resuspended in acid medium. The obvious disappearance of mesohaem from the pH 8 medium during the 4 h incubation and the dark coloration of the algal cells even after transfer to acid medium suggested that

the cells had indeed taken up mesohaem as found previously for protohaem (Brown *et al.*, 1981).

Dark-grown cells that had been exposed to pH 8 medium (with or without mesohaem or protohaem addition) were washed twice with water and resuspended in acid medium containing 1.8% (w/v) glucose and 0.16% (w/v) 5-aminolaevulinic acid hydrochloride (Troxler *et al.*, 1978). When appropriate, 5-amino[4- ^{14}C]laevulinic acid was added at this stage. The molar quantities of this material were insignificant compared with the amount of unlabelled aminolaevulinate. The suspension was incubated at 37°C in the dark for 3–5 days and the resulting excreted phycocyanobilin was extracted into chloroform and evaporated to dryness under a stream of N_2 as described previously (Brown *et al.*, 1981).

Incubation of *C. caldarium* with mesohaem in the light

Dark-grown cells (5 ml packed cell volume) that had been exposed to pH 8 medium (with or without mesohaem addition) were washed twice with water and resuspended in 100 ml of medium at pH 1.7. Such cultures were exposed to light (approx. 5400 lx) at 37°C (Brown *et al.*, 1981). Greening became evident after about 12 h, indicating phycocyanin and chlorophyll *a* synthesis. After 60–64 h total illumination, the cells were collected by centrifugation (bench centrifuge for 10 min) and the supernatant was retained. The cells were washed with fresh acid medium, collected by centrifugation and the supernatant was combined with that collected as described above. The cells were further washed with 0.1 M-potassium phosphate buffer (pH 7) and the supernatant was retained. Each supernatant fraction was extracted (three times) with an equal volume of chloroform and the chloroform extracts were evaporated to dryness under a stream of N_2 .

The cells were resuspended in 0.1 M-potassium phosphate buffer (pH 7) and disrupted by using a French pressure cell (American Instrument Co., Inc., Silver Springs, MD, USA) at 2.21×10^5 kPa (32 000 lbf/in 2) internal pressure. After centrifugation (36 000 g for 30 min) phycocyanin was partially purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$, precipitated with trichloroacetic acid and its chromophore (phycocyanobilin) was removed and isolated as described previously (Troxler *et al.*, 1978; Brown *et al.*, 1981).

Purification of pigments

The bilins obtained either by excretion from cells (in the dark and in the light) or by cleavage from apoprotein synthesized in the light, were converted into the dimethyl esters by refluxing with 7% (w/v) BF_3 in methanol (Brown *et al.*, 1981). The pigments were initially purified by t.l.c.

on silica-gel G plates, developed with chloroform/methyl acetate (2:1, v/v). This system (system 1) has been used to purify phycocyanobilin dimethyl ester (R_F 0.73) and another derivative, phycocyanobilin dimethyl ester-methanol adduct (R_F 0.60), which is formed from phycocyanobilin either during methylation or during cleavage from apoprotein (Beuhler *et al.*, 1976; Brown *et al.*, 1981). When appropriate, the band of pigment corresponding to phycocyanobilin dimethyl ester was further purified by t.l.c. on silica-gel G plates developed with chloroform/ethyl acetate/cyclohexane (32:9:4, by vol.). This system (system 2) has been used to separate mesobiliverdin isomers (Colleran, 1971).

Other measurements

Radiochemical assay of pigments was carried out as previously described (Brown *et al.*, 1981). Mass-spectral measurements were carried out on an AEI MS9 mass spectrometer, and electronic spectra were determined on a Unicam SP.8-100 spectrophotometer in stoppered cuvettes.

Results and discussion

Incubation of mesohaem with C. caldarium in the dark

Two approaches were made to assess the possible role of mesohaem in the biosynthesis of phycocyanobilin in the dark. In the first, where unlabelled mesohaem and ^{14}C -labelled 5-amino-laevulinate were administered to *C. caldarium* cells, incorporation of mesohaem would be indicated by a decrease in the specific radioactivity of excreted phycocyanobilin compared with that of a control sample given ^{14}C -labelled 5-amino-aevulinate but not mesohaem. In the second approach, cells containing radiolabelled mesohaem were incubated with unlabelled 5-amino-laevulinate. In these experiments no control is necessary and incorporation would be indicated by labelling of the excreted phycocyanobilin.

Table 1 shows data from three experiments using the first approach. Significant quantities of blue pigment were excreted into the suspending medium in both the control cultures and the cultures containing unlabelled mesohaem. In experiments 1 and 2 (Table 1), much more pigment was present in the dimethyl ester band from incubations with cells containing mesohaem than from cells of control incubations. When the pigments excreted by cells to which mesohaem had been administered were purified by t.l.c. (system 1 described above), bands corresponding to both phycocyanobilin dimethyl ester and phycocyanobilin dimethyl ester-methanol adduct were observed. Absorption maxima for these pigments (in

chloroform) are shown in Table 1, indicating that the pigments formed in the presence of mesohaem appear to be the same as those formed in the control sample. However, the wavelength of maximum absorption of the long-wavelength band (approx. 625 nm) appeared to be red-shifted compared with authentic phycocyanobilin dimethyl ester (600 nm). Table 1 also shows the ratio of the measured radioactivity per millilitre to absorbance for each sample counted. For phycocyanobilin dimethyl ester, the ratio for the cells to which mesohaem was administered is considerably less than that for control cells. However, the corresponding ratios for phycocyanobilin dimethyl ester-methanol adduct do not show substantial differences between control cells and those to which mesohaem had been administered. One possible explanation of this apparent anomaly could be the presence of an unlabelled impurity in the phycocyanobilin dimethyl ester band from cells containing mesohaem. This possibility was tested by subjecting a sample of the suspected mixture to further t.l.c. with a different elution system (system 2; see the Experimental section). After three developments with this system, the sample separated into two distinct blue bands, confirming the presence of an impurity in the phycocyanobilin dimethyl ester. (The control phycocyanobilin dimethyl ester band did not separate into two bands on t.l.c. in system 2.) The use of an authentic sample as marker showed that the upper band corresponded to phycocyanobilin dimethyl ester. Preliminary liquid-scintillation counting showed that most of the radioactivity was contained in this upper band. This result implied that the impurity (lower band) was unlabelled and therefore was not derived from phycocyanobilin or synthesized *de novo* in any other way from 5-aminolaevulinate. It therefore seemed likely that the blue impurity was derived from the unlabelled mesohaem, and that it was probably mesobiliverdin IX α (see Fig. 1).

The mass spectrum of the impurity yielded a strong molecular ion at m/z 614. This corresponds to the M_r of both phycocyanobilin dimethyl ester and mesobiliverdin dimethyl ester. However, the mass spectrum of phycocyanobilin dimethyl ester shows a strong fragment peak at m/z 599, corresponding to loss of a methyl group (Troxler *et al.*, 1978), a feature that is not present in the mass spectrum of mesobiliverdin dimethyl ester (Beuhler *et al.*, 1976). There was no strong peak at m/z 599 in the observed spectrum and this adds support to the probable identity of the impurity as mesobiliverdin. The electronic spectrum of the impurity in chloroform showed maxima at 368 nm and 630 nm with a ratio of absorbances at these wavelengths of 3.4. The corresponding literature values (Stoll & Gray, 1977) for mesobiliverdin IX α dimethyl ester

Table 1. *Effect of mesohaem on the incorporation of 5-amino[4-¹⁴C]laevulinate into phycocyanobilin in the dark*
The volume of each incubation mixture was 40 ml. Where appropriate, mesohaem was pre-incubated with cells of *C. caldarium* as described in the Experimental section. The pigments were obtained by t.l.c. in solvent system 1. The absorbance and radioactivity values refer to solutions of purified pigment recovered from t.l.c. plates, of which 1 ml was used for radioactivity counting.

Phycocyanobilin derivative	Mesohaem added (mg)	Radioactivity added (μ Ci)	λ_{max} (nm)	Absorbance	Radioactivity (d.p.m./ml)	$10^{-4} \times$ Radioactivity/absorbance (d.p.m./ml)
Experiment 1*						
Dimethyl ester	0	10	371	0.320	3472	1.09
Dimethyl ester	4.0	10	369	1.962	5652	0.29
Methanol adduct	0	10	371	1.118	10263	0.92
Methanol adduct	4.0	10	369	1.257	10303	0.82
Experiment 2						
Dimethyl ester	0	5	370	0.154	871	0.57
Dimethyl ester	2.0	5	370	1.370	2285	0.17
Methanol adduct	0	5	373	0.640	3396	0.53
Methanol adduct	2.0	5	373	0.355	2183	0.61
Experiment 3*						
Dimethyl ester	0	10	369	0.231	2484	1.08
Dimethyl ester†	4.0	10	—	—	—	—
Methanol adduct	0	10	373	1.994	19264	0.97
Methanol adduct	4.0	10	373	0.516	4940	0.96

* In order to increase total yield of pigment, two identical cultures (40 ml each) containing mesohaem were incubated simultaneously and combined after pigment synthesis.

† Since only a small amount of pigment was recovered in this experiment, it was all retained for use in the second t.l.c. separation (see Table 2).

Table 2. *Specific radioactivities of pigments excreted by C. caldarium in the dark, obtained after second t.l.c.*
In order to calculate the specific radioactivity of the 'mixture band', it was assumed that the molar absorption coefficient in chloroform at 369 nm was the same as that of phycocyanobilin dimethyl ester [i.e. $54.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Brown *et al.*, 1981)]. For mesobiliverdin IX α dimethyl ester, $\epsilon_{369} = 54.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Stoll & Gray, 1977). In the control experiments, no mesohaem was added.

Sample	$10^{-10} \times$ Sp. radioactivity (d.p.m./mol)		
	Expt. 1	Expt. 2	Expt. 3
Control phycocyanobilin dimethyl ester	58.8	30.6	58.4
Mixture band	15.5	9.0	*
Purified phycocyanobilin dimethyl ester	37.5	22.8	42.2
Mesobiliverdin IX α dimethyl ester	6.2	3.4	12.8

* Since only a small amount of pigment was recovered in this experiment, it was all retained for use in the second t.l.c. separation.

are 369 nm and 631 nm (absorbance ratio 3.46) and for phycocyanobilin dimethyl ester the values are 369 nm and 600 nm (absorbance ratio 2.8). This

further supports the assignment of the impurity as mesobiliverdin IX α dimethyl ester. Finally an authentic sample of mesobiliverdin IX α dimethyl ester co-chromatographed with the lower band but not with phycocyanobilin dimethyl ester on t.l.c. (system 2). This system also distinguishes mesobiliverdin IX α from other mesobiliverdin isomers. These results therefore confirm that significant quantities of mesobiliverdin IX α are formed when mesohaem is incubated with *C. caldarium* and that the initial purification produces a band on t.l.c. that is a mixture of the dimethyl esters of phycocyanobilin and mesobiliverdin IX α .

Table 2 shows results from the three experiments (see Table 1) in which *C. caldarium* cells were incubated with unlabelled mesohaem and 5-amino[4-¹⁴C]laevulinate in the dark and in which the initial mixture band was resolved by a second t.l.c. Specific radioactivities were calculated from literature values for molar absorption coefficients (Stoll & Gray, 1977; Brown *et al.*, 1981). It is clear that in each case the mixture band has a considerably lower specific radioactivity than the control phycocyanobilin dimethyl ester band, but that this is resolved on further t.l.c. into a purified phycocyanobilin dimethyl ester band that has a specific radioactivity closer to that of the control and a mesobiliverdin band with a relatively low specific radioactivity. It appears, however, that even a

Table 3. Incubation of *C. caldarium* containing ^{14}C -labelled protohaem or ^{14}C -labelled mesohaem with 5-aminolaevulinate in the dark

The volume of each incubation mixture was 40 ml. T.l.c. was carried out as described in the Experimental section.

Sample	Amount counted for radioactivity (nmol)	Radioactivity (d.p.m.)	$10^{-10} \times \text{Sp. radioactivity (d.p.m./mol)}$
Cells incubated with protohaem (sp. radioactivity 28.6×10^{10} d.p.m./mol)			
Phycocyanobilin dimethyl ester	5.10	262	5.1
Cells incubated with mesohaem (sp. radioactivity 28.6×10^{10} d.p.m./mol)			
Mixture band (from first t.l.c.)	5.26	1308	24.9
Phycocyanobilin dimethyl ester (after second t.l.c.)	0.58	23	*
Mesobiliverdin IX α dimethyl ester (after second t.l.c.)	21.3	6045	28.4
Cells incubated with mesohaem† (sp. radioactivity 21.0×10^{10} d.p.m./mol)			
Mixture band (from first t.l.c.)	9.7	1689	17.4
Mesobiliverdin IX α dimethyl ester (after second t.l.c.)	9.3	1747	18.8

* The radioactivity was so close to background level that the specific radioactivity could not be accurately determined.

† The phycocyanobilin dimethyl ester obtained after the second t.l.c. was insufficient for determination of specific radioactivity.

second t.l.c. is insufficient to purify either component completely.

Experiments were carried out by the second approach, in which a culture containing ^{14}C -labelled protohaem (known to be incorporated into phycocyanobilin under these conditions; Brown *et al.*, 1981), was used along with two cultures containing ^{14}C -labelled mesohaem. The results are shown in Table 3, together with the specific radioactivity values for protohaem and mesohaem. Results for phycocyanobilin dimethyl ester-methanol adduct (not shown) revealed little radioactivity from the cultures containing mesohaem but significant radioactivity from that containing protohaem. In the latter culture, incorporation of label from protohaem into phycocyanobilin dimethyl ester is clearly shown (as expected from previous data). The proportion of phycocyanobilin obtained from the added haem (about 20%) is also consistent with previous findings (Brown *et al.*, 1981). The mixture bands obtained from the first t.l.c. of pigment from the cultures containing radiolabelled mesohaem also contained significant radioactivity. However, after a second t.l.c., carried out as described above, resolution into two bands again occurred. The band corresponding to phycocyanobilin dimethyl ester contained little pigment and very little radioactivity (from one culture the amount recovered was insufficient for analysis), whereas the lower band, corresponding to mesobiliverdin IX α dimethyl ester, was relatively intense and contained significant radioactivity. Moreover, the specific radioactivity of this material was very similar to that of the labelled

mesohaem administered. These data from ^{14}C -labelled mesohaem therefore complement and confirm the data from experiments with ^{14}C -labelled 5-aminolaevulinate.

Incubation of C. caldarium containing mesohaem in the light

When cells containing mesohaem were incubated in the light, as described in the Experimental section, it was immediately evident that, in addition to the expected greening of the cells, a blue-green pigment was excreted into the suspending medium. No such pigment was excreted in control cultures (without mesohaem addition) or in cultures to which protohaem had been administered (Brown *et al.*, 1981). After extraction into chloroform and methylation, this excreted pigment was shown to be mesobiliverdin IX α by the same spectroscopic and chromatographic criteria used previously. In addition, washing of the cells (separated from the supernatant by centrifugation) with phosphate buffer, pH 7, resulted in the release of considerably more pigment. This was also shown to be mesobiliverdin IX α . When ^{14}C -labelled mesohaem was used, the results obtained from liquid-scintillation counting of both the mesobiliverdin excreted into the suspending medium and that obtained by washing the cells with phosphate buffer, pH 7, are shown in Table 4. These results show that the pigments have specific radioactivities very similar to that of the mesohaem administered, and confirm that they are derived from this mesohaem.

Table 4. Incubation of cells containing ^{14}C -labelled mesohaem in the light
 ^{14}C -labelled mesohaem (sp. radioactivity 15.3×10^{10} d.p.m./mol) was pre-incubated with *C. caldarium* as described in the Experimental section.

Sample	Amount counted for radioactivity (nmol)	Radioactivity found (d.p.m.)	$10^{-10} \times$ Sp. radioactivity (d.p.m./mol)
Mesobiliverdin dimethyl ester (excreted into medium)	26.6	3734	14.0
Mesobiliverdin dimethyl ester (from pH 7 wash)	23.6	3120	13.2
Phycocyanobilin dimethyl ester (after first t.l.c.)	17.3	133	0.77
	17.3	128	0.74
Phycocyanobilin dimethyl ester (after second t.l.c.)	9.4	19	Negligible
	9.4	23	Negligible

The cells from this experiment were disrupted and phycocyanobilin was purified (by using a single t.l.c. separation) as previously described (Brown *et al.*, 1981). When samples were counted for radioactivity, the methanol adduct sample contained no significant radioactivity above background, but samples of the phycocyanobilin dimethyl ester revealed small but significant radioactivity, as shown in Table 4. Since this could have been due to contamination by a small quantity of radiolabelled mesobiliverdin IX α dimethyl ester, the remainder of the phycocyanobilin dimethyl ester sample was subjected to the second t.l.c. step (system 2) as described above. Although no obvious separation into two bands occurred (any mesobiliverdin dimethyl ester present would be expected to form a lower band), a sample from the phycocyanobilin dimethyl ester band was taken and two portions of this were analysed for radioactivity. These results, shown in Table 4, reveal insignificant radioactivity over background, confirming that the second t.l.c. had eliminated most of the radioactive impurity and that phycocyanobilin itself is unlabelled.

General discussion

These experiments have clearly shown that mesohaem is not an intermediate in the biosynthesis of phycocyanobilin. Although a partially reduced intermediate cannot be ruled out, the present results therefore support the idea that the macrocyclic ring of protohaem itself is cleaved to yield biliverdin and that the two reduction steps occur subsequently. This idea is also supported by preliminary results in which biliverdin administered to *C. caldarium* cells becomes incorporated into phycocyanobilin (S. B. Brown & J. A. Holroyd, unpublished work) and studies that show that, under certain conditions, biliverdin may be formed by *C. caldarium* (Köst & Benedict, 1982). To this extent, therefore, the biosynthesis of plant bilins appears to follow the same pathway as the

well-known route for production of mammalian bile pigments.

Two additional conclusions may be deduced from the present work. The first is that, although mesohaem is not incorporated into phycocyanobilin, it is undoubtedly degraded by *C. caldarium* cells to mesobiliverdin IX α . This may be taken as further evidence for the existence of an algal haem oxygenase, for which, apparently, mesohaem can act as a substrate. This finding is comparable with results from studies on mammalian haem oxygenase, which show that mesohaem is readily accepted as a substrate, though it is less effective than the natural substrate, protohaem (Schmid & McDonagh, 1975; Yoshida *et al.*, 1981).

A further conclusion from the present study is that mesobiliverdin IX α is not an intermediate in phycocyanobilin biosynthesis. This would be possible in principle, even if mesohaem were not a precursor, since biliverdin could be reduced to mesobiliverdin as shown in Fig. 1. However, since mesobiliverdin is formed *in situ*, clearly it must be well placed for potential incorporation so far as its subcellular location is concerned. The fact that it is not incorporated shows that it cannot be an intermediate.

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