Biliverdin IXα, Intermediate and End Product of Tetrapyrrole Biosynthesis

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Dark-grown cells of the unicellular rhodophyte *Cyanidium caldarium* were incubated with 17 mmol/l 5-aminolevulinic acid in the dark. The excreted pigments were extracted with chloroform and butanol. The presence of biliverdin IXα in the chloroform-extract (besides phycocyanobilin and other pigments) was demonstrated using TLC, HPLC and chromic acid degradation. A pathway leading to phycocyanobilin is discussed. A green pigment from egg shells of *Turdus merula* (black bird) was also identified as biliverdin IXα with small amounts of protoporphyrin IX, using the same methods as above.

Introduction

Cyanobacteria (the former blue-green algae), rhodophyta and cryptophyta (golden-brown algae) contain phycocyanins and phycoerythrins, special light-harvesting pigments, which have been identified as pigment-protein complexes with covalently linked bile pigment chromophore(s) (*e.g.* [1–5]; for reviews see [6–8]). Although the chemical nature of the prostetic groups phycocyanobilin (in phycocyanins) and phycoerythrobilin (in phycoerythrins) as bile pigment is known for many years [9–14] their biosynthetic pathway is still not fully understood [15–24] or more or less uninvestigated (phycoerythrobilin).

Recent investigations yielded the following sequence of biosynthesis matching that of bile pigment synthesis in animals (compare [25], here older literature) up to the stage of biliverdin. Starting point is 5-aminolevulinic acid (ALA) which may be obtained as a product of different biosynthetic routes for example by action of the enzyme ALA-synthetase (ALAS) from succinyl-CoA and glycin (*e.g.* [26–29], from glutamate (*e.g.* [27–30]) or dioxovaleric acid (*e.g.* [26, 31]). Two molecules of ALA are condensed to porphobilinogen (PBG) [32] which in turn reacts to protoporphyrin IX via uroporphyrinogen [33, 34], 7-,6-,5-carboxylic porphyrinogen, coproporphyrinogen (4-carboxylporphyrinogen) [35, 36], 3-carboxylic porphyrinogen [37] (Fig. 1) (also compare: [38]).

At the stage of protoporphyrin, the biosynthesis branches to the magnesium-porphyrin pathway (chlorophyll) and the heme (= iron porphyrin) pathway. Brown *et al.* [24] could recently show that labeled heme is incorporated into phycocyanin (not chlorophyll) of the red alga *Cyanidium caldarium* what proves that the biosynthetic pathway is leading via the iron-porphyrin-branch rather than via the magnesium-porphyrin-branch.

So far, intermediates between protoheme (= iron-protoporphyrin) and phycocyanobilin have not been found. One such intermediate, concluding from the bile pigment synthesis in animals, should be biliverdin IXα. The following experiments with the red alga *Cyanidium caldarium* furnish proof that biliverdin is indeed such an intermediate.

In egg shells of *Turdus merula* (black bird) biliverdin was found to be a biosynthetic end product. In animals, the pool of available iron is generally low; most of the iron is "fixed" in iron-porphyrins (*e.g.* hemes) as the central atom. It can be reclaimed by ring splitting (*via* heme oxygenase) [25]). The ironless, oxidized open chained reaction product, biliverdin IX, mostly the α-isomer (biliverdin IX γ was found in *Pieris brassicae* [39]) is excreted, often in reduced form (bilirubin: gall stones, rats bile; stercobilin: faeces [40]) or deposited in the animal, for example insects [39, 41], spiders [42] and others [43, 44] or egg shells as already reported 1931/32 by Lemberg [45, 46] for *Larus ridibundus*. Often, protoporphyrin IX is also found in egg shells [47]. A further example is presented in this paper.

Abbreviations: ALA, 5-aminolevulinic acid; hR_f, $100 \times R_f$; PCB, phycocyanobilin.

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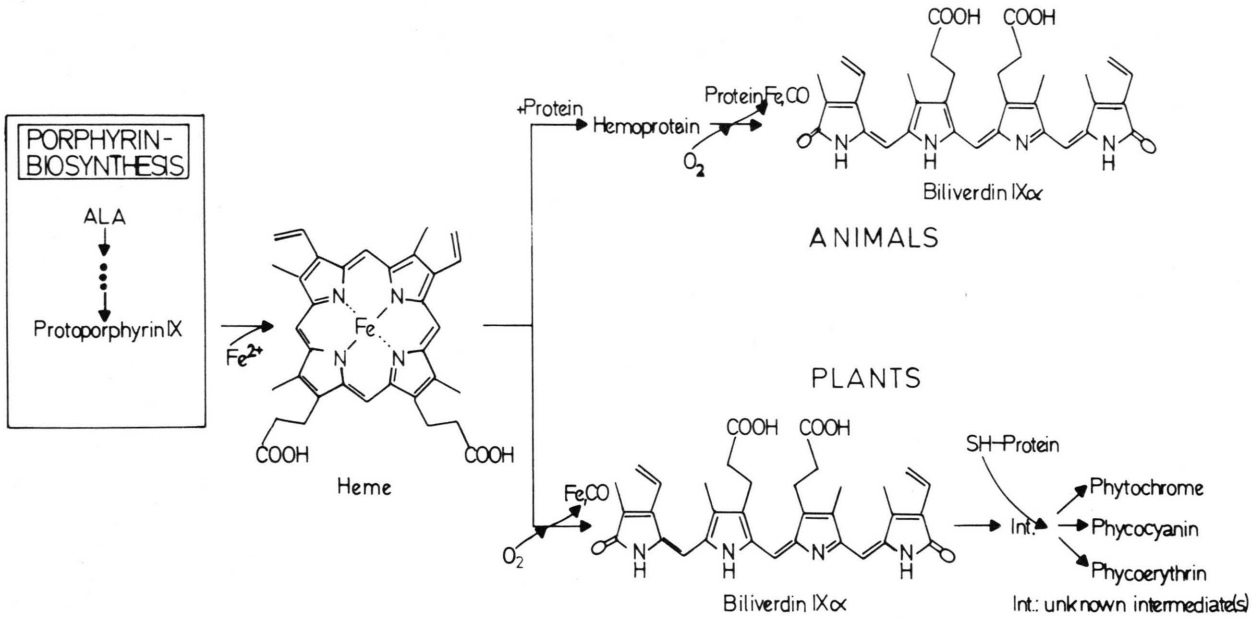

Fig. 1. Biosynthetic pathway leading to biliverdin IX α (animals) and phycobiliproteins (plants) with biliverdin IX α as intermediate. Plantal biliproteins are possibly formed by addition of an HS-Protein to as yet unknown intermediate(s). Also compare Fig. 5.

Materials and Methods

Biliverdin excreted from Cyanidium caldarium

Cultures of *Cyanidium caldarium* strain III-D-2* (2 × 1 l) were kept at 22 °C for 8 days in a glucose based medium (adapted from [48]) under steady shaking to a density of 9×10^9 cells/ml. After that time, the cells were harvested by centrifugation (30 min/10000 rpm) under sterile conditions, and taken up in 2 × 200 ml medium with 17 mmol/l 5-ALA. After 72 h the cells were separated by centrifugation (20 min/16800 rpm). The blue-coloured medium was acidified with hydrochloric acid to a final concentration of 2% and extracted thrice with chloroform (100 ml total volume). The still intensely coloured water phase was extracted with an equal volume of butanol. After filtration, the chloroform phase and the butanol phase were brought to dryness (evap. rot., 40 °C) and the dry product esterified with 6% (w/v) methanolic sulfuric acid (10 ml, 7 h, − 19 °C). The esterification mixture was treated with chloroform/water, the separated chloroform phase dried by filtration and brought to dryness. The dry product was taken up in acetone and used for the following experiments.

* Obtained through courtesy of Dr. R. F. Troxler.

Biliverdin from egg shells of Turdus merula

Fresh material was collected during May. Prior to the actual extraction, the egg shells were washed with chloroform. Typically, shells from one egg were ground in a mortar with 10 ml of 6% (w/v) methanolic sulfuric acid and the resulting slurry kept for 8 h at 4 °C under steady stirring. After that time, undissolved material was removed by centrifugation and the resulting green supernatant worked up with chloroform/water.

Chromatography

TLC

Silicagel 60 coated HPTLC plates (10 × 10 cm) from E. Merck, Darmstadt, were used. After application of the mixture to be separated, the plates were developed with one of the following solvent systems:

1. hexane-butanone-2-glacial acetic acid = 10:5:1;
2. carbon tetrachloride-ethyl acetate = 1:1;
3. benzene-petrol ether (40–60 °C)-methanol-ethyl acetate = 48.5:40.0:10.5:9.0.

As reference standards porphyrin esters (*e.g.* protoporphyrin IX dimethylester) from Porphyrin Products, Logan were applied, also for mixed chromatograms. Samples of biliverdin IX (α, β, γ, δ) were a gift of Dr. H. Scheer, München.

HPLC

HPLC was carried out on a Waters apparatus: Pump: model 6000 A, Injector U6K, Detector: Spektromonitor II, Latek, Heidelberg, set at 650 nm. Straight column, length: 250 mm; 4.6 mm I.D. Material: stainless steel (Waters). Solvent system: methanol/water = 78:22. Column packing: μ Bondapak C_{18} 10 μ (Waters, Königstein).

Degradation experiments

Chromic acid degradation of bile pigments was performed as described by Rüdiger [49] (degradation directly on the TLC-plate).

Results and Discussion

Absorption spectra were taken from neutral chloroform solutions. The spectrum of the extracted egg shell pigments exhibited maxima at 505, 540, 576, 630 nm respectively 378, 660 nm, which favorably compare with those of protoporphyrin IX respectively biliverdin IXα. The spectra of the chloroform and butanol extract of *C. caldarium* medium showed a maximum at ≈ 370 nm and a broad band at ≈ 600 nm. A shoulder at 660 nm (biliverdin) is only found in the spectrum of the chloroform extract.

Further more convincing evidence arose from chromatography on HPTLC plates in different solvent systems. Here, a green spot chromatographs with the same R_f-values as biliverdin IXα; it did not separate in a mixed chromatogram. Since it was possible in our systems to clearly separate biliverdin IXα from the other isomers and protoporphyrin IX (see Table I) the green pigment was identified as biliverdin IXα.

The separation of the pigment from the medium extract of *C. caldarium* was hampered by the fact that it contained phycocyanobilin (with a nearly identical R_f-value to biliverdin in most systems). However, in the system benzene-petrol ether (40–60 °C)-methanol-ethyl acetate = 48.5:40.0:10.5:9.0 (Fig. 2) and hexane-butanone-2-glacial acetic acid = 10:5:1 a clear separation on HPTLC plates occured, using medium extracts of several independent algal cultures in different experiments.

Table I. HPTLC: bile pigments and protoporphyrin.

Layer	L_1	L_1	L_1	L_2
Solvent	S_1	S_2	S_3	S_4
Technique	T_1	T_1	T_1	T_2
Detection	D	D	D	D
Literature	+	+	+	[53]
Compound[a]	hR_f			
Biliverdin IXα	40[b]	38[b]	53[b]	16[c]
Biliverdin IXβ	44	43	57	51[c]
Biliverdin IXγ	45	42	57	37[c]
Biliverdin IXδ	43	31	51	68[c]
Phycocyanobilin	36	39	55	–
Protoporphyrin IX	52	65	79	–

[a] compound as dimethylesters;
[b] bile pigments investigated chochromatograph with biliverdin IXα;
[c] values deduced from [53].

Layer L_1: Silicagel 60 HPTLC plates (E. Merck, Darmstadt); L_2: Silicagel G.
Solvent S_1: hexane-butanone-2-glacial acetic acid = 10:5:1; S_2: carbon tetrachloride-ethyl acetate = 1:1; S_3: benzene-petrol ether (40–60 °C)-methanol-ethyl acetate = 48.5:40.0:10.5:9.0; S_4: *n*-heptane-butanone-2-glacial acetic acid = 10:5:1.
Technique T_1: analytical; ≈ 200 μg bile pigment/spot; 1 run with solvent system indicated (multiple development does not improve separation); T_2: 3 runs with solvent system indicated.
Detection D: visual observation.
+ This paper.

We further characterized the pigment as biliverdin IXα by HPLC. There, a mixture of biliverdin IXα, β, γ, δ and phycocyanobilin is separated by using water/methanol = 22:78 as eluent [50].

A prominent feature of biliverdin (Fig. 1) is given by the two vinyl side chains of ring A and D yielding 3-methyl-2-vinyl-maleimide (Fig. 3a) upon chromic acid degradation which can be distinguished from 3-methyl-2-ethyl-maleimide (Fig. 3b) by its fluorescence under UV-light.

In our case, chromic acid degradation was carried out directly on thin-layer plates following a separation of the individual pigments (from the egg shells of *T. merula* and the algal medium extract, respectively). The result is given in Fig. 4.

A chlorine-tetramethyl-benzidine-positive spot at $hR_f = 62$ (fluorescent under UV-light prior to application of the reagent) proved the presence of 3-methyl-2-vinyl-maleimide (MVM). MVM is not found if pure phycocyanobilin is degraded. Instead,

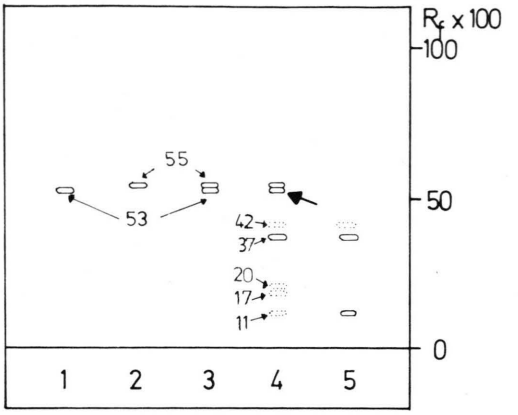

Fig. 2. Separation of biliverdin IXα dimethylester and phycocyanobilin dimethylester. Layer: Silicagel 60 (HPTLC plate, E. Merck, Darmstadt) Solvent system: benzene-petrol ether (40–60 °C)-methanol-ethyl acetate = 48.5: 40.0:10.5:9.0. 1: biliverdin IXα dimethylester (standard, green spot on plate); 2: phycocyanobilin dimethylester (standard, blue spot on plate); 3: 1 + 2; 4: chloroform extract of *Cyanidium caldarium* medium. After solvent removal the pigments had been esterified. Arrow indicates green spot running identical with biliverdin IXα. At $hR_f = 37$, a polar, as yet unidentified blue pigment was observed; 5: butanol extract of *Cyanidium caldarium* medium (after previous chloroform extraction) (esterified pigments). Biliverdin and phycocyanobilin are lacking, the blue bile pigment at $hR_f = 37$ is the main component. 4, 5: dashed circles indicate minor spots.

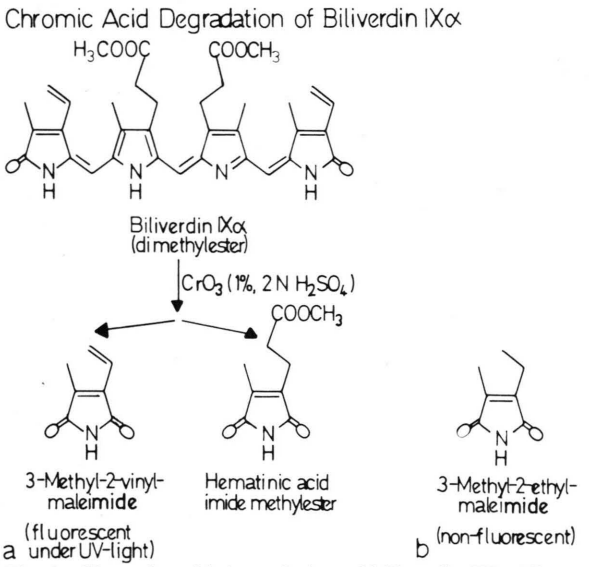

Fig. 3. Chromic acid degradation of biliverdin IXα (dimethylester). Chromic acid degradation of biliverdin from *T. merula* and *C. caldarium* yields 3-methyl-2-vinyl-maleimide (MVM) (fluorescent under UV-light, contrary to 3-methyl-2-ethyl-maleimide which does not fluorescence) and hematinic acid imide (as methylester) (HSE). The imides were visualized by UV-light (MVM) or chlorine/tetramethyl benzidine reagent after separation on HPTLC plates (see Fig. 4).

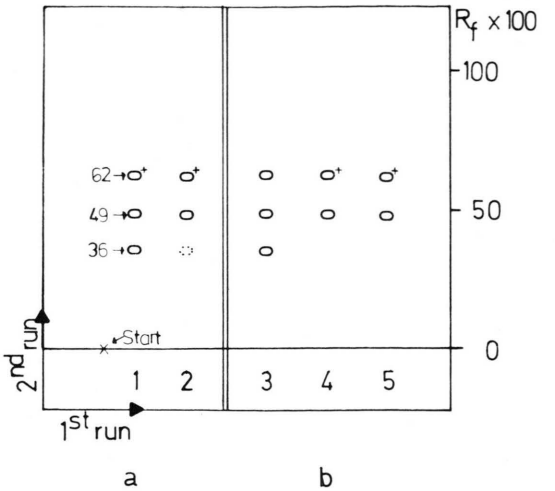

Fig. 4. a: Separation of chloroform extract of medium from ALA-incubated *C. caldarium* cells with subsequent chromic acid degradation. Layer: Silicagel 60 (HPTLC plate, Merck, Darmstadt). 1st run: solvent system A: benzene-petrol ether (40–60 °C)-methanol-ethyl acetate = 48.5: 40.0:10.5:9.0. 2nd run: solvent system I: carbon tetrachloride-ethyl acetate-cyclohexane = 10:3:1; Spots were visualized with the chlorine/tetramethyl benzidine reagent. 1st run: Separation of chloroform extract of *C. caldarium* medium (compare Fig. 2 lane 4). Two main products, a polar bile pigment at $hR_f = 37$ and phycocyanobilin/biliverdin at $hR_f = 55/53$ were obtained. The spots were treated with chromic acid (1% in 2 N H_2SO_4) and chromatographed in the second dimension. 2nd run: "lane" 1: Chromic acid degradation of polar bile pigment. Imides obtained: 3-methyl-2-vinyl-maleimide ($hR_f = 62$) fluorescent under UV-light; hematinic acid methylester ($hR_f = 49$); 3-methyl-2-ethylidene-succinimide ($hR_f = 36$) (MES). For phycocyanobilin + biliverdin, only traces of MES were obtained. Note fluorescence at $hR_f = 62$ (+). b: Lane 3–5 without previous run in solvent system A. Spots were applied on the plate, immediately degraded with chromic acid and chromatographed in solvent system I (for imides, see a). 3: chromic acid degradation of phycocyanobilin dimethylester (reference); 4: biliverdin IXα dimethylester (reference); 5: biliverdin IXα dimethylester from egg shells of *T. merula*. Note fluorescence at $hR_f = 62$ indicating presence of 3-methyl-2-vinyl-maleimide (+).

3-methyl-2-ethylidene-succinimide and 3-methyl-2-ethyl-maleimide are found (usually in low yield).

The main pigment excreted by *C. caldarium* cells upon incubation with ALA, however, is neither PCB [15, 17] nor biliverdin which appears in comparably small amounts, but rather a more polar blue bile pigment yielding upon chromic acid degradation considerable amounts of 3-methyl-2-vinyl-maleimide, 3-methyl-2-ethyl-maleimide, hematinic acid and a more polar imide with the R_f of *E*-2-ethylidene-3-methyl-succinimide.

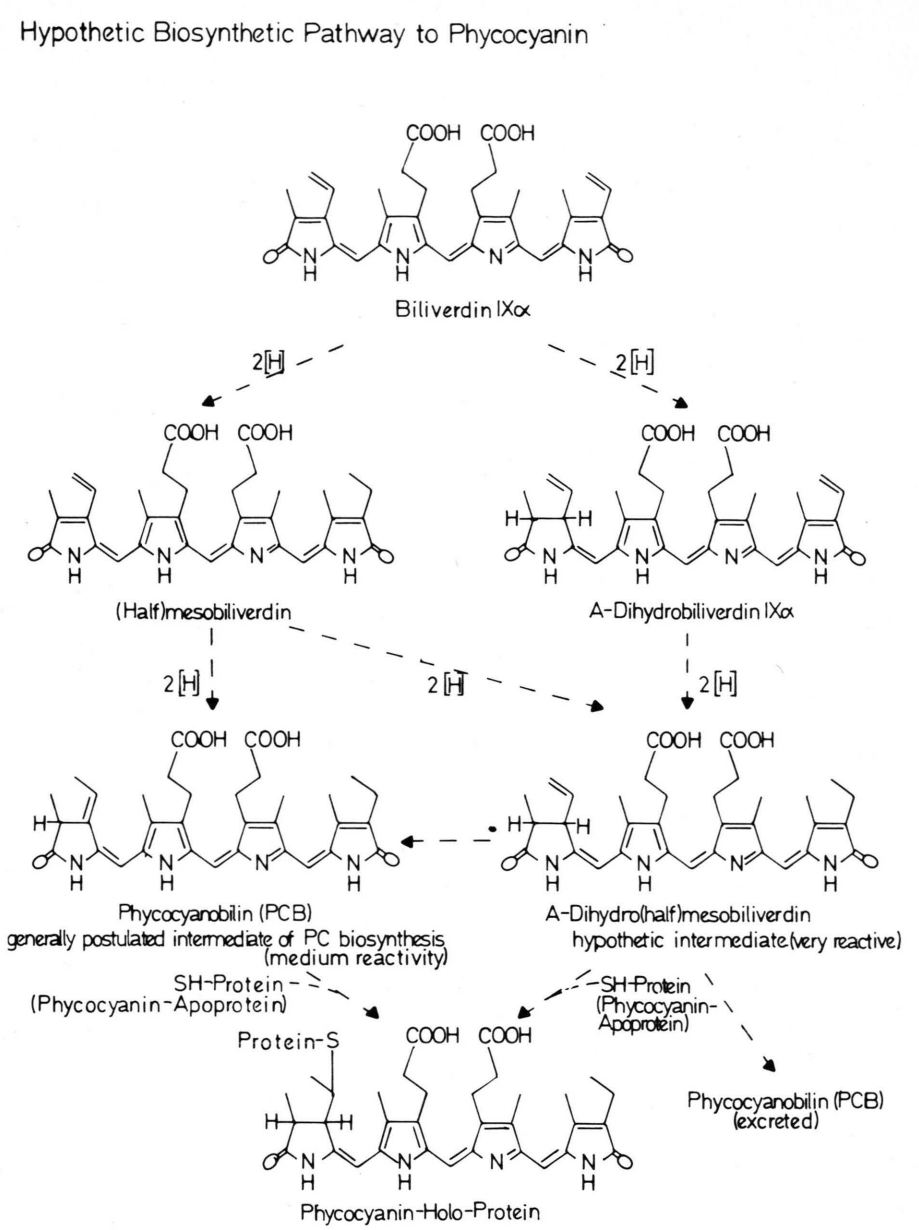

Fig. 5. Hypothetic pathway to phycocyanin. The intermediate from porphyrin biosynthesis, biliverdin IX α (see Fig. 1) is stepwise hydrogenated at the vinyl side chain of the D-ring and the A-ring nucleus, leaving a reactive vinyl side chain. This (hypothetical) intermediate adds SH-protein (phycocyanin-apoprotein) to form phycocyanin. Some PCB is formed as a conversion product of the intermediate (migration of double bond).

Concluding from recent results [51] and experiments described by Troxler [15], the chloroform extract of the algal medium (after incubation of *C. caldarium* with 17 mmol/l ALA) contains all the biosynthetic intermediates from ALA up to protoporphyrin to be expected namely 8-,7-,6-,5-,4-,3-,2-carboxylic porphyrins (protoporphyrin IX), biliverdin and phycocyanobilin together with more polar blue pigments. The butanol extract of the medium (which was pre-extracted with chloroform) contains no biliverdin but rather polar blue pigments [51].

Pathway to phycocyanin

Biliverdin is obtained from protoporphyrin IX (after iron insertion and oxidative ring opening/iron removal [25] and a key pigment of the phycocyanobilin pathway (Fig. 1, 5)).

Biliverdin IX α, the precursor of the phycocyanin chromophore and therefore also phycocyanobilin* differ in the number of double bonds. PCB (Fig. 5) possesses an ethyl side chain at ring D instead of a vinyl group, the hydrogenated ring A is bearing an ethylidene group. Therefore, two double bonds must have been hydrogenated, most probably stepwise. Intermediates may be either A-dihydrobiliverdin or (half) mesobiliverdin with one ethyl and one vinyl side chain (at ring A). From the latter compound, a 1-4-hydrogenation of the butadiene configuration at ring A might lead directly to phycocyanobilin which in turn may add the SH-group of the apoprotein to form the thioether linkage in α-position of the side chain. PCB, however, is regarded as much less reactive than A-dihydro(half)-mesobiliverdin. However, PCB may be formed from the latter probably very unstable compound by migration of a double bond. Which compound is the correct precursor of phycocyanin biosynthesis (that is, adds HS-apoprotein) is still open; the question will be answered by an isolation of the correct pigment(s)* and/or feeding experiments similar to those recently conducted by Brown *et al.* [24] and will require the use of labeled (^{13}C, ^{14}C, ^{15}N etc.) substrates like A-dihydrobiliverdin IX.

The more polar bile pigment also excreted by *C. caldarium* is under investigation. Studies are under way to isolate and identify the proposed intermediate pigments (see Fig. 5) between biliverdin and phycocyanobilin.

The general validity of the pathway (Fig. 1) for the animal kingdom, too is substantiated by a further proof of biliverdin (together with protoporphyrin IX, its precursor) in egg shells.

Acknowledgements

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* PCB can be splitt off from the biliprotein, *e.g.* by methanolysis (see lit. [52], here also older literature).

* From the medium after incubation of *C. caldarium* or other algae with ALA.

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