# Rubins and Rubinoid Addition Products from Phycocyanin *

W. Kufer and H. Scheer

Botanisches Insitut der Universität München, Menzinger Straße 67, D-8000 München 19

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The verdin-type chromophore of denatured C-phycocyanin (**1**) from *Spirulina platensis* is reduced to the corresponding rubin (**2 a**) by sodium borohydride. The structure assigned is in agreement with the uv-vis spectroscopic properties of the product and was deduced from model studies with free bile pigments.

Analogous model studies using sodium dithionite demonstrated a two-fold reactivity for this reagent, leading to products which are both of the rubin spectral type under the conditions tested. True rubins (10,22-dihydrobilindions) are formed in low yield only if an excess of reagent is used in methanol/water mixtures. It is accompanied by polar addition product(s) of the same spectral type, which are generally formed exclusively. In particular, no bilirubin was formed under the reaction conditions previously applied for the chemical modification of phycobiliproteins and phytochrome. From this finding and from the strikingly different properties of the borohydride and dithionite products, of phycocyanin upon renaturation, the dithionite product is suggested to be a rubinoid addition product (**2 b**) rather than a hydrogenation product.

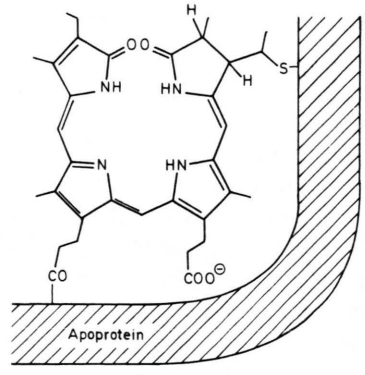
In contrast to the dithionite addition product **2 b** of phycocyanin, the chromophore of the true phycorubin (**2 a**) remains stable upon renaturation. The uv-vis spectral properties of the chromophore are not markedly different whether the apoprotein is in its native or denatured state. The different electrophoretic mobilities of native (renatured) phycocyanin compared to the renatured borohydride product suggest that these two have different protein conformations.

The preparation of these phycorubins renders the extensive techniques of bilirubin chemistry applicable in the study of biliproteins.

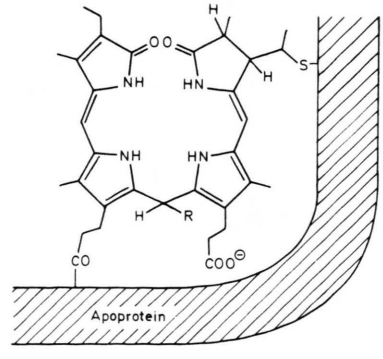
## Introduction

Bile pigments, open-chain tetrapyrrolic compounds, serve important functions in plants. In contrast to mammalian bile pigments derived from heme degradation, they are biosynthesized as photoreceptors which are covalently bound to apoproteins, thus forming "biliproteins" (for a recent review, see [1]). Two functionally different groups can be distinguished: Firstly the phycobiliproteins as light harvesting pigments of photosynthesis in cyanobacteria, red and cryptophytan algae, and secondly phytochrome and the phycochromes as photomorphogenetic receptor pigments of higher plants and some algae.

The physico-chemical properties of the bile pigment chromophores are profoundly influenced by the native proteins. This is mainly due to non-covalent protein chromophore interactions which are essential in biliproteins to optimize the proper-




**1**




**2a** R = H **2c** R = $SO_3^-$
**2b** R = $SO_2^-$ **2d** R = S – $CH_2$ – $CH_2OH$

*Abbreviations:* PC, phycocyanin; tlc, thin layer chromatography; mob, electrophoretic mobility relative to standard.

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Reprint requests to Prof. Dr. H. Scheer.

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ties of the chromophores in their function as photoreceptors. This can be easily seen from a comparison of native and (reversibly) denatured pigments (see ref. [1] for a survey). Recently, we developed a technique for the selective chemical modification of biliprotein chromophores [2, 3]. The experiments reported here have been performed to yield more detailed information about the structure of the chemically modified products, and the noncovalent protein chromophore interactions in biliproteins. The previous experiments of this type were carried out with the reducing agents dithionite, sulfite and 2-mercaptoethanol, and pigments of the rubinoid spectral type were obtained [3]. Here, we wish to report the reaction of C-phycocyanin (**1**) (C-**PC**) and some free bilins as model compounds with sodium borohydride. This reagent has been used [4, 5] for selective reduction of bilindiones ("biliverdin" type chromophores like (**3**)) to 10,22-dihydrobilindiones ("bilirubin" type chromophores like (**4**))*.

## Results

*Reaction of phycocyanin with sodium borohydride*

The reactions are summarized in Scheme 1. When PC (concentration range 30–130 μM chromophores**) denatured by 8 M urea in 50 mM sodium phosphate buffer, pH 7.5, was treated at 0 °C with solid sodium borohydride (25–40 mM***), the bands in the uv-vis spectrum at 602 nm ($\varepsilon = 15400$ per chromophore) and 355 nm ($\varepsilon = 37000$) [3] disappeared completely after 20–30 min and a new band at 416 nm arose simultaneously. The pH of the solution increased to about 9, which could be avoided without significant changes in reactivity by using a buffered solution of sodium borohydride. Using PC (48 μM chromophores) and 13 mM $NaBH_4$, a 90% decrease of the 602 nm band was observed within 30 min. The reaction went to completion within additional 30 min after a further addition of the same amount of borohydride (Fig. 1)*. Assuming a 1:1 stoichiometry with no by-product formed, $\varepsilon_{416} = 24200$ (per chromophore) was calculated from the spectral changes, and with $\varepsilon_{602} = 15400$ [3] for the educt chromophores**. The vis-spectrum remained unaltered when the reducing agent was removed by gel filtration on a column equilibrated with 8 M urea. The yellow colour of the borohydride product also remained unchanged after simultanous removal of urea and borohydride by gel filtration ($\lambda_{max} = 418$ nm). The extinction coefficient of the pigment thus obtained was determined by unfolding the protein again, by addition of solid urea to a final concentration of 8 M. With $\varepsilon = 24200$ as reference value for the denatured pigment (see above) and allowing for the dilution caused by addition of urea, $\varepsilon_{418} = 21900$ was obtained for "native"phycorubin.

The electrophoretic mobility of the product obtained after removal of urea and borohydride differed from that of native PC in tris-glycine electrophoresis. With equal protein concentrations applied, the yellow product had a higher mobility (mob = 0.72 *vs.* bromophenolblue as reference compound) as compared to PC (mob = 0.59) (Fig. 2). They were identical, however, in SDS-gel electrophoresis having a single band corresponding to MW = 19900 in coelectrophoresis using the system of Weber and Osborn [6] (a separation into subunits was generally not obtained with PC from *Spirulina platensis*). Reaction of the yellow pigments with diazotized ethyl anthranilate (5–10 fold molar excess) yielded uv-vis spectroscopically identical pigments with a long wavelength band at 485 nm, irrespective of the removal of only $NaBH_4$ or both $NaBH_4$ and urea before the reaction.

Addition of solid sodium borohydride (43 mM) to native PC (6.6 μM chromophores, calculated from $\varepsilon_{620} = 98700$/chromophore [3]) led to only partial conversion to the yellow pigment. After 10 min reaction

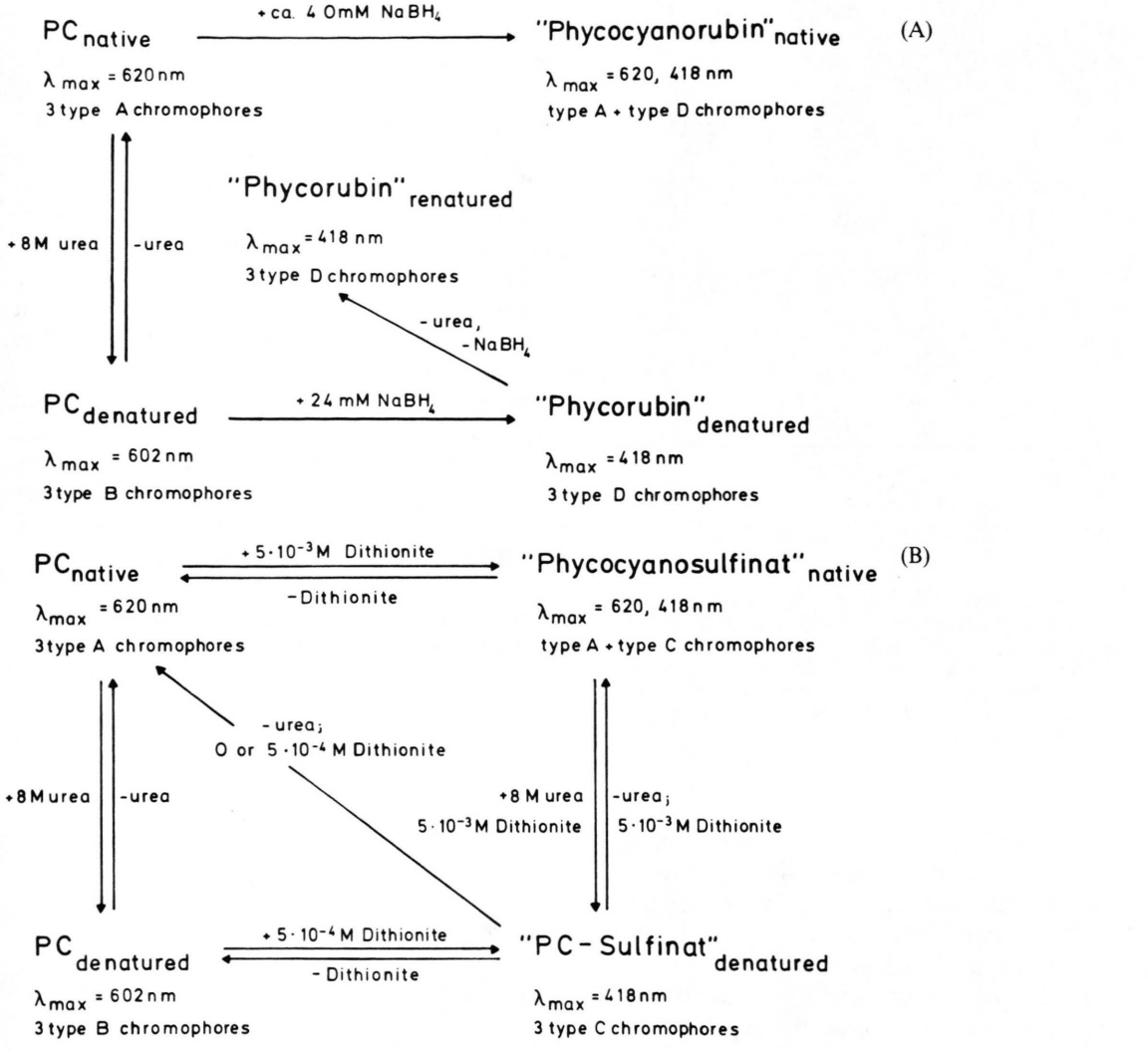
* See ref. [43] for the nomenclature of bile pigments, and formulas for the numbering system. In addition, the non-systematic terms "rubin" and "verdin" are used to characterize pigments bearing the conjugation systems of bilirubin and biliverdin, respectivley.

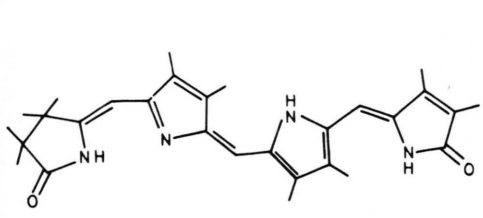
** Concentrations throughout are given for chromophores. Since monomeric ($\alpha_1\,\beta_1$) C-**PC** contains three chromophores, the protein concentration is one third of the chromophore concentration.

*** $NaBH_4$ is unstable in protic solvents. All concentrations given are the initial concentrations of the reagent.

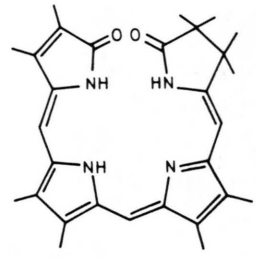
* Addition of a larger excess of $NaBH_4$ results in a further reaction of the product ($\lambda_{max} = 416$ nm) to products not absorbing in the visible spectral range. It is thus recommended to carry out the reaction with a minimum amount of borohydride added in successive portions, and to follow the reactions spectrophotometrically.

** The extinction coefficients for the reaction products of denatured C-**PC** with sodium dithionite, sodium sulfite and 2-mercaptoethanol have been determined by the same method to 25500, 27900 and 23600 $l \cdot M^{-1}$, respectively [3].

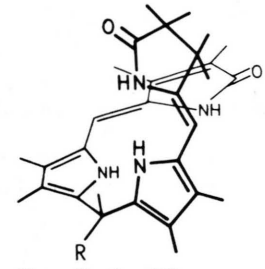


Type A



Type B



Type C: R = $SO_2^-$
Type D: R = H

Scheme 1. Reaction scheme of the reversible renaturation of PC from *Spirulina platensis*, and of the reactions with sodium borohydride (A) and sodium dithionite (B). The chromophore structures A – D are schematic representations of the chromophore geometry. Structure A is representative of an extended conformation without major steric hindrance of the *β*-pyrrolic substituents. Structure B is the cyclic-helical conformation found for biliverdin in solution [22, 23]. Structures C and D have been drawn with the two dipyrromethenone units nearly perpendicular to each other, similar to the crystal structure of bilirubin [46, 47]. The reactions of PC with sodium sulfite and 2-mercaptoethanol principally follow Scheme 1B as well but different concentrations of the reagents are needed.

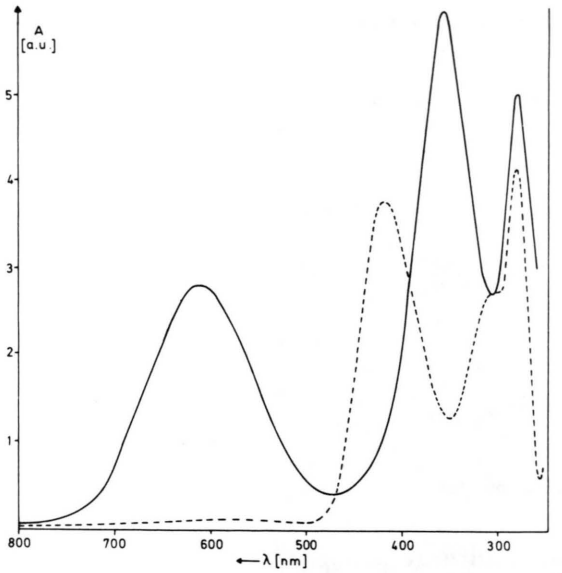



Fig. 1. Absorption spectra of denatured phycocyanin (48 μM) in phosphate buffer (50 mM, pH 7.5) containing urea (8 M) (——), and of the same solution after addition of $NaBH_4$ in the same solvent (- - -). The borohydride was added in two equal portions corresponding to a nominal final concentration of 24 mM, at times 0 and 30 min, the spectrum was recorded at $t = 60$ min.

time at 0 °C, the long wavelength absorption of native PC at 620 min decreased by 48%. The shape of the band was changed, being more strongly bleached at the short wavelength side. At the same time, a shoulder appeared around 410 nm at the uv-band of the chromophores. If the concentration of $NaBH_4$ was raised to 0.63 M, the pigment was completely bleached within 20 min, without absorption maxima in the visible range.

*Reaction of model compounds for the PC chromophore with sodium borohydride and sodium dithionite*

The results are summarized in Table I. Treatment of biliverdin (**3a**)** in methanol ($7\times10^{-5}$ M) with solid sodium borohydride for 5 min at ambient temperature yielded a yellow product with $\lambda_{max}=435$ nm. The spectral properties (solvent shifts, see Table I) were identical with bilirubin (**4a**), and cochromatography gave a single spot. Mixing of equal volums of a methanolic biliverdin solution (final conc. $2\times10^{-5}$ M with an aqueous sodium borohydride solution (final conc. 0.6 M) led to a mixture of bilirubin with its III- and XIII-α isomeres (**5a**, **6a**) as identified by tlc with several appropriate solvent systems (a, e, f in Table I)*. The reduction to **4a** proceeded smoothly, too, if solid $NaBH_4$ was added to a solution of **3a** in phosphate buffer (50 mM containing 8 M urea), but no isomers were found in this case.

Results analogous to those obtained with the free acid **3a** were obtained with biliverdin dimethylester (**3c**) and also for mesobiliverdin (**3b**) (see Table I). The products were again identified as the corresponding rubins by cochromatography with authentic samples in different solvent systems, and in the case of **4b** also by $^1$Hmr (data in experimental part).

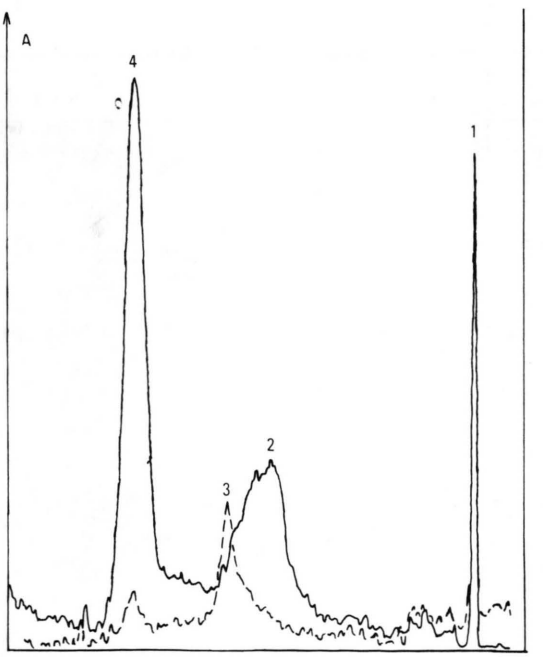



Fig. 2. Densitogram of polyacrylamide-gels after electrophoresis of phycocyanin (——, band 2) and renatured phycorubin (- - -, band 3). The gels were unstained and scanned with detection wavelengths of 609 and 417 nm, respectively. Band 1 corresponds to the origin, band 4 to bromophenol-blue used as front marker.

* Three different tlc systems have been described [7a–c] for the separation of the III-, IX- and XIII-α isomers of bilirubin. We found a fourth system, silicagel developed with benzene/absolute ethanol (25:2 v/v) very suitable for this purpose. This system had originally been described for the separation of dialkylesters of bile pigments [7d]. Moreover, also the corresponding isomers of mesobilirubin (**4b**, **5b**, **6b**) are separated with this system; they cochromatograph with the solvent system $CHCl_3$/acetic acid (99:1, v/v).

** Formulas of the model compounds see p. 186.

Table I. Vis-spectral and tlc-data of model compounds for the chromophore of denatured PC, their products with reducing agents, and of authentic rubins (10,22-dihydrobilindions).

| Compound and reagent | Absorptions $\lambda_{max}$ [nm] | $\lambda_{max}$ after extraction in $CHCl_3$ | tlc: $R_f$-values ×100 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | f | g | h |
| Biliverdin, **3a** | 655–668[i], 678[j], 654[k], 663[l], 675[m] | | 01 | | | 05 | 08 | 00 | 88 | |
| **3a** + $NaBH_4$[i] | 435 | 445 | 65[p] | | | | | | | |
| **3a** + $NaBH_4$[j] | 451, 418 sh | 450 | 76[q]<br>85<br>92 | | | 00 | 66[q]<br>79 | 83[q]<br>91<br>96 | 65 | |
| **3a** + $NaBH_4$[m] | 445 | 446 | 75[p] | | | | | 63[p] | | |
| **3a** + dithionite (1 mM)[j] | 415, 436 sh | 416 | 04 | | | | | | | |
| **3a** + dithionite (24 mM)[j] | 405, 432 sh | 454 | 57[n,q]<br>62<br>67 | | | 00 | 41[n,q]<br>50<br>60 | | [o] | |
| **3a** + dithionite (.2 mM)[m] | 403, 425 sh | 442 | 00 | | | | | | | |
| **3a** + dithionite (20 mM)[m] | 415 | 442 | 00 | | | | | | | |
| **3a** + dithionite (.2 mM)[l] | 398, 425 | 449 | 00 | | | | | | | |
| **3a** + dithionite (20 mM)[l] | 417 | 446 | 00 | | | | | | | |
| **3a** + sulfite (40 mM)[j] | 403, 444 sh | 405 | 00 | | | 00 | | | 88 | |
| **3a** + 2-mercaptoethanol (2.5 M)[i,s] | 436, 416 sh | | | | | | | | | |
| Bilirubin, **4a** | 449, 418 sh[j], 454[k] | | 62–85 | | | 00 | 60–80 | 34–91 | 65 | |
| Mixture of III, IX and XIII α-isomers of bilirubin (**5a**, **4a**, **6a**) | | | 68–76 (**6a**)<br>77–85 (**4a**)<br>86–92 (**5a**) | | | 00 | 60–77<br>60–80<br>62–87 | 30–83<br>34–92<br>42–96 | 65 | |
| Biliverdin-dimethylester, **3c** | 666[i], 672[j], 662[k] | | 49 | 38 | 11 | 20 | 27 | 16 | 21 | |
| **3c** + $NaBH_4$[j] | 459, 414 sh | 408, 442 sh | 27, 37 | 02 | 07 | 06 | 51 | 19 | 07 | |
| **3c** + dithionite (.5 mM)[j] | 445, 420 sh | 406, 438 sh | 00, 04 | 00 | 00 | 00 | 04 | 00 | 64 | |
| Bilirubin-dimethylester, **4c** | 450, 418 sh[i], 458, 417 sh[j] 403, 436 sh[k] | | 37 | 02 | 07 | 06 | 51 | 19 | 07 | |
| Mesobiliverdin, **3b** | 644[i], 657–670[j] | | 01 | | | 03 | | 80 | | |
| **3b** + dithionite (30 mM) | 398, 414 sh | | 66[t,r] | | | 69[r] | | 65 | | |
| **3b** + sulfite (20 mM) | 393, 420 sh | | 04[t] | | | 02[t] | | 90 | | |
| **3b** + 2-mercaptoethanol (2.5 M)[i,s] | 422 | | | | | | | | | |

[a]) Silica, developed with benzene/ethanol = 25 : 2 (see footnote p. ■).
[b]) Silica, developed with chloroform/aceton = 95 : 5 [33].
[c]) Silica, developed with toluene/95% ethanol = 95 : 5. This system was used for HPLC of the III, IX and XIII a isomers of bilirubindimethylester [44].
[d]) Polyamide, developed with methanol/water = 3 : 1 [7d]).
[e]) Silica, developed with toluene/acetic acid/water = 5 : 5 : 1, upper phase.
[f]) Silica, developed with chloroform/acetic acid = 99 : 1 [7d]).
[g]) Polyamide, developed with methanol/10% ammonia/water = 9 : 1 : 2 [7d]).
[h]) Silica, developed with carbon tetrachloride/aceton = 9 : 1.
[i]) Methanol.
[j]) Methanol/water = 1 : 1.
[k]) Chloroform.
[l]) 50 mM sodium phosphate buffer, pH 7.5.
[m]) Same buffer containing 8 M urea, pH 7.5.
[n]) Not identified residue at starting point.
[o]) $R_F$ undetermined due to extensive tailing.
[p]) Identical to **4a** in cochromatography.
[q]) Identical to isomer mixture **4a**, **5a**, **6a** in cochromatography.
[r]) Identical to **4b** in cochromatography.
[s]) Unstable during work-up with carbon tetrachloride/water, unstable against acid [3].
[t]) Additional not identified by-products.
[u]) Increased $R_f$-values were found, when the plates were reused, due to incomplete removal of acetic acid from the adsorbent.

Table I (continued).

| Compound and reagent | Absorptions $\lambda_{max}$ [nm] | $\lambda_{max}$ after extraction in $CHCl_3$ | tlc: $R_f$-values ×100 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | f | g | h |
| Mesobilirubin, **4b** | 424, 398 sh[j]), 419[m]) | | 66–74 | | | | 60–69 | 31 | 65 | |
| Mixture of III, IX and XIII α-isomers of mesobilirubin (**5b, 4b, 6b**) | | | 68<br>74<br>78 | | | | 60 | 31 | | |
| Octaethylbilindion, **7** | 646[i]) | | | | | | | | | 47 |
| **7** + $NaBH_4$[i]) | 400, 420 sh | | | | | | | | | 11 |
| **7** + 2-mercaptoethanol[i,s]) | 426 | | | | | | | | | |
| 2,3-Dihydrooctaethyl-bilindion, **8** | 596[i]) | | | | | | | | | 65 |
| **8** + $NaBH_4$[i]) | 400 | | | | | | | | | 05 |
| **8** + dithionite[j]) | 398[c]) | | | | | | | | | |
| **8** + 2-mercaptoethanol[i,s]) | 410 | | | | | | | | | |

The octaethylbilindion **7** and its 2,3-dihydro-derivate **8** similarily yielded yellow products upon treatment with solid borohydride in methanol. **7** reacted smoothly at ambient temperature, **8** only at elevated temperatures (50 °C, under nitrogen). The conjugation system of **8** is identical to that of C-PC.

Using sodium dithionite instead of borohydride, the results depended critically on the reaction conditions. In an experiment in methanol/water (1:1, v/v) analogous to the one described above, a yellow product different from **4a** was obtained from **3a** with 1 mM dithionite. It migrated with an $R_F$-value of or close to zero on silica gel-tlc, developed with neutral or acidic solvent systems (a, e, f in Table I). On polyamide-tlc with a basic solvent system, it migrated close to the front (g in Table I). No bilirubin (**4a**) could be detected under these conditions. Upon rising the dithionite concentration to 24 mM, **4a** and its isomers (**5**, **6**) could be identified as by-products besides the aformentioned polar product(s). The product mixture was analyzed quantitatively after reaction on the preparative scale (**3a** = $10^{-4}$ M, dithionite = $2 \times 10^{-2}$ M). After work up, esterification with diazomethane and preparative tlc, **4c** was isolated in 4% yield and identified by uv-vis and tlc comparison with authentic bilirubin dimethylester (**4c**). The results obtained with mesobiliverdin (**3b**) were analogous (see Table I).

In another series of experiments, biliverdin was treated with dithionite in the absence of organic solvents. In particular, the reaction of **3a** ($9 \times 10^{-6}$) was studied in phosphate buffer (50 mM sodium phosphate, pH 7.5) containing urea (8 M), *e.g.* under conditions identical with those used for the reaction of PC [3]. With dithionite concentrations ranging from $2 \times 10^{-4}$ to $2 \times 10^{-2}$ M, no bilirubin could be identified in the yellow product mixtures. Chromatography revealed only polar product(s) ($R_F = 0$ in system a, as compared to 0.85 for **4a**). The same results were obtained using buffer without urea.

The conformationally restricted pigments phorcabilin (**9**) and isophorcabilin (**10**) reacted smoothly, too, with dithionite (see Table II). Treatment of **9** ($13 \times 10^{-6}$ M) with sodium dithionite ($5 \times 10^{-5}$ M) in a buffer/methanol mixture led to decrease of the

Table II. UV-vis spectral data of the biliverdin **11**, the verdins **9** and **10** with an extended conformation similar to the one suggested for the chromophores of native PC [25], and of their reaction products with reducing agents.

| Compound | $\lambda_{max}$ [nm] in methanol |
|---|---|
| Phorcabilin-dimethylester, **9** | 555<br>562[b]) |
| **9** + $NaBH_4$ | 417 |
| **9** + dithionite (0.05 mM) | 412[b]) |
| Isophorcabilin-dimethylester, **10** | 606, 690 (sh)<br>614[b]) |
| **10** + $NaBH_4$ | 417 |
| **10** + dithionite (0.5 mM) | 435, 455 (sh),<br>405 (sh)[b]) |
| Biliverdin IX γ-dimethylester, **11** | 645 |
| **11** + $NaBH_4$ | 408 |
| **11** + dithionite[b]) | [c]) |

[a]) See footnote (s), Table I.
[b]) 50 mM sodium phosphate buffer, pH 7.5/methanol = 1:1
[c]) Not determined due to precipitation.

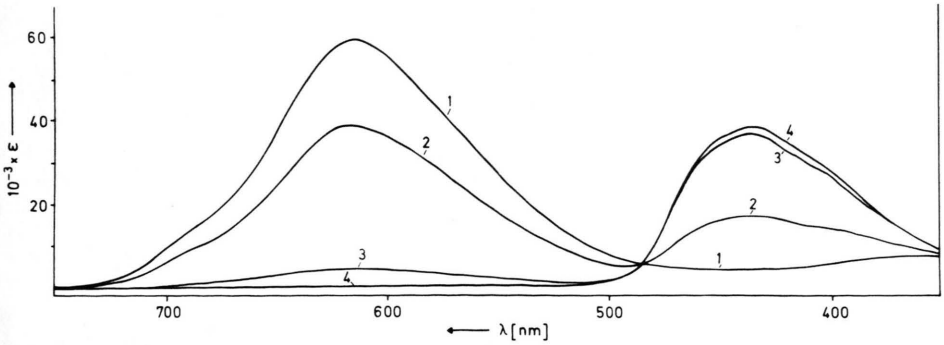



Fig. 3. Titration of isophorcabilindimethylester (**10**) ($9.6 \times 10^{-6}$ M) in a 1:1 mixture of methanol and phosphate buffer (50 mM, pH 7.5) with sodium dithionite. The absorption spectra were recorded at the following concentrations of dithionite curve 1: 0 M, 2: $5 \times 10^{-6}$ M, 3: $5 \times 10^{-5}$ M, 4: $5 \times 10^{-4}$ M, and have been recorded each 5 min after the respective additions of the reagent.

long-wavelength band ($\lambda_{max} = 562$ nm) by 95%, and the appearance of only one product band ($\lambda_{max} = 412$ nm). Titration of **10** with dithionite (Fig. 3) led to 96% bleaching with $5 \times 10^{-4}$ M dithionite, again forming a yellow product ($\lambda_{max} = 435$, shoulders at 435 and 405 nm).

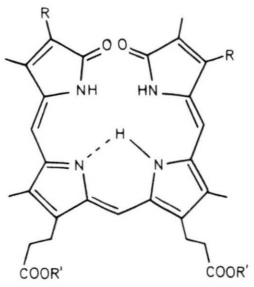
In addition to the results described here, some earlier results [3] of the reaction of model compounds for the PC chromophore with sodium sulfite and 2-mercaptoethanol have been incorporated in Table 1. They have already been published, together with preliminary results obtained with sodium dithionite.

## Discussion

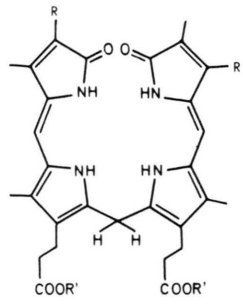
The reaction product of denatured PC with sodium borohydride has an absorption maximum at 416 nm. Spectroscopically similar products were found with other reducing agents, viz. sodium dithionite, sodium sulfite and 2-mercaptoethanol [2, 3] (see also Scheme 1). This suggests for all these products an interruption of the conjugated system of the PC chromophore (**1a**) at the C-10 position. For the rings C and D, this leads to the chromophoric system of mesobilirubin (**4c**, $\lambda_{max} = 419$ nm in phosphate buffer containing 8 M urea). All four products have similar extinction coefficients: $\varepsilon_{416} = 24200$ with borohydride, $\varepsilon_{418} = 25500$ with dithionite [3], $\varepsilon_{418} = 27900$ with sulfite and $\varepsilon_{418} = 23600$ with 2-mercaptoethanol. They amount to roughly 50% of the value reported for mesobilirubin (**4c**) ($\varepsilon_{433}$ ($CHCl_3$) = 54600 [10]); thus supporting the general structure **2**. For rings A and B, the interruption of the conjugation at C-10 leads to a vinylpyrrole chromophore not absorbing in the visible range (*e.g.* 313 nm for a series of 3,4-dihydro-6(1H)-pyrromethenons [8, 9] closely related to the ring A, B-fragment of **2a**). The borohydride product has an absorption in this spectral range ($\lambda_{max} = 310$ nm) which appears as a shoulder of the protein band ($\lambda_{max} = 280$ nm), the other products could not be investigated in this region due to strong absorption of the reagents.

Although the phycocyanin reaction products with these four reductants are uv-vis spectroscopically nearly identical, they differ in their structure concerning the residue R in formula **2**. From uv-vis and tlc-data of the reaction products of model compounds with sulfite, and from the acid reversible product formation with 2-mercaptoethanol, the structures **2c** and **2d** were suggested [3] for these pigments arising from addition of the respective reagents. The pronounced reactivity of C-10 in bilindiones towards nucleophiles had been inferred first from MO calculations [12]. After circumstantial evidence [3, 13a, b], NMR-data of adducts of thioles with bilindiones in agreement with the proposed structure **2d** have been reported [14, 15], and the thermodynamic data for related equilibria have been obtained [15].

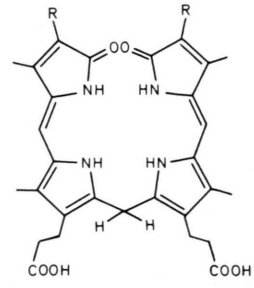
Reaction of the same model compounds with sodium borohydride led via addition of hydride at C-10 to true reduction products, *e.g.* bilirubins, as revealed by comparison of their uv-vis and tlc-data with those of the authentic corresponding rubins (see Table I). Reduction of verdins to rubins with borohydride in methanol has been reported in the



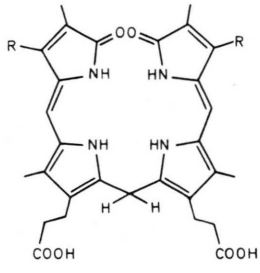
**3a** R = $C_2H_3$ R′ = H
**3b** R = $C_2H_5$ R′ = H
**3c** R = $C_2H_3$ R′ = $CH_3$



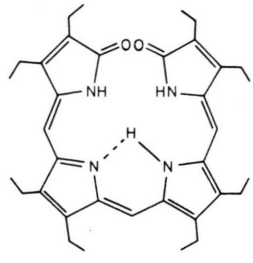
| | R = | R′ = |
|---|---|---|
| **4a** | $C_2H_3$ | H |
| **4b** | $C_2H_5$ | H |
| **4c** | $C_2H_3$ | $CH_3$ |



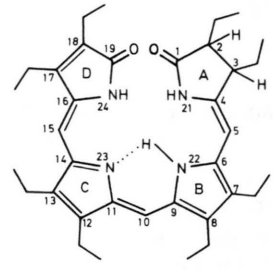
**5a** R = $C_2H_3$
**5b** R = $C_2H_5$



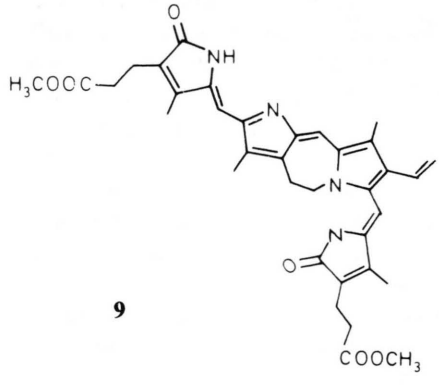
**6a** R = $C_2H_3$
**6b** R = $C_2H_5$



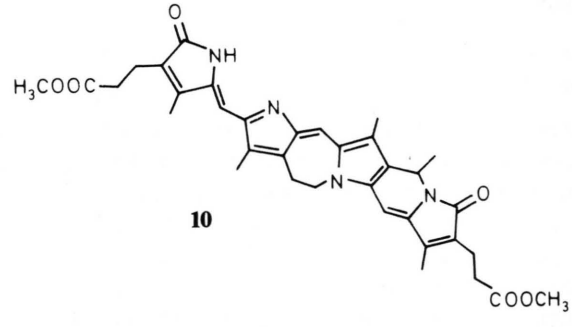
**7**



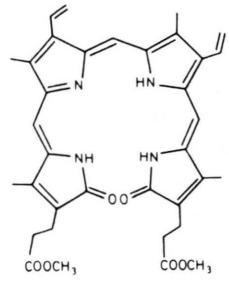
**8**



**9**



**10**



**11**

literature [4, 5]. The appearance of III- and XIII-$\alpha$ isomers **5** and **6** besides bilirubin (**4a**) and mesobilirubin (**4c**) in methanol/water can be explained by the isomerisation ("scrambling") reaction, which has been observed with bilirubin in moderately basic aqueous solutions [11]. Structure **2a** was thus assigned to the reaction product of phycocyanin with borohydride, and since it is a true hydrogenation product is should be named "phycorubin".

Conflicting results have been reported for the reaction of verdins with dithionite. Bilirubin has been isolated in low yield from the reaction of sodium dithionite with biliverdin, in aqueous alcaline solution [16], and the reduction of a biliverdin-iodine complex to bilirubin has been reported [17]. On the other hand, a product different from bilirubin has been identified as well [18]. The model studies reported here support this differential reactivity of bilindiones towards dithionite. In the neutral pH-range studied, rubins have only been identified in low yield, and only in solutions containing methanol. In particular, rubins have in no case been observed under the conditions used for the reactions of PC with sodium dithionite. A product with similar chromatographic mobilities as the sulfite product (see Table I) arose instead. Derivatives of nicotinamide are reduced by dithionite in a complex mechanism. Addition of sulfinate, the homolytic fission product of dithionite, has been suggested as the first step, followed by a slower and pH-dependent elimination and a final true reduction step with $HSO_2^-$ rather than $S_2O_4^-$ as the reagent proper for hydride transfer [19]. Whereas this sequence eventually leads to reduction of nicotinamide in good yields, this is apparently not the case with bilindiones, possibly due to the increased stability of the first addition products*. Even prolonged treatment of the product at pH 2.7 during workup did not result in bilirubin formation. Structure **2b** is thus suggested for the dithionite reaction products of PC and APC, and analogous structures for the reaction products with other biliproteins, *e.g.* phycoerythrin, and phytochrome $P_r$. They had earlier been tentatively identified as true reduction products [2, 3], which has to be corrected in view of the comparison with the rubins proper obtained with borohydride. Thus, the stability of phycorubin (**2a**) upon renaturation (removal of urea *e.g.* by gel filtration), was strikingly different as compared to the products obtained with sulfite (**2c**), 2-mercaptoethanol (**2d**) and dithionite (**2b**) [3] see Scheme 1. The yellow colour of the borohydride product remained unchanged, while the products with sulfite, 2-mercaptoethanol and dithionite were converted back to native PC.

The reactions summarized in Scheme 1 B, can readily be explained by a reversible addition to the central methine bridge, with the equilibrium strongly dependent on the state of the protein. In the denatured state with the chromophores still bound but uncoupled from the protein, the addition-elimination equilibrium is shifted to the addition product, whereas the educt is favored in the native or renatured pigments even in the presence of a large excess of dithionite. Also, the equilibrium is differentially affected by the different environments of the chromophores of identical molecular structure (*e.g.* three in PC). Reversible addition reactions are thus a useful tool to probe these different environments [3].

The different stabilities of the native and denatured pigments may originate from a different conformational freedom of the chromophores in the two states. Biliverdin dimethylester (**3b**) and related bilindions are rather flexible molecules, which exist in solution in several rapidly interconverting forms [21] of predominantly cyclic conformation [22, 23]. The nearly identical spectroscopic properties of denatured biliproteins make a similar mobility likely for their chromophores. The comparably narrow absorption bands of native biliproteins, as well as their high fluorescence yield indicate that the chromophores are rigidly fixed in (an extended [24, 25]) conformation. The nucleophilic addition results in the conversion from an $sp^2$ to $sp^3$ hybridised C-10, and thus necessarily a conformational change of the chromophores. A conformational fixation could then shift the equilibrium between the biliprotein chromophore proper, and its addition product. We have attempted to test this hypothesis by studying the reaction of phorcabilin-dimethylester (**9**) and

* Caughey and Schellenberg [20] have identified sulfinate addition products of nicotinamides by $^1$Hmr spectroscopy. Attempts to identify the corresponding product of biliverdin failed. **3c** in $C^2HCl_3$ (0.02 M) was treated with an aqueous solution of sodium dithionite, and the yellow solution formed was thoroughly washed with water and dried. The solution exhibited only broad signals. When the reaction was carried out with sodium borohydride instead, the spectrum of the product was identical to that of bilirubin dimethylester (**4c**) (see experimental part).

isophorcabilin dimethylester (**10**), bilins held in an extended conformation by additional bridges between the pyrrole rings. Unfortunately, however, the central seven membered ring is still flexible enough to allow both $sp^2$- and $sp^3$-hybridization of C-10 without steric restrictions. Thus, **9** and **10** react with both dithionite and borohydride as well as does biliverdin-IX $\gamma$-dimethylester **11** (see Table II).

The slow formation of denatured PC from the dithionite product [3] after removal of the reducing agent (gel filtration, see Scheme 1a) is then explained by a shift in the equilibrium to the side of phycocyanin.

According to the structure of the phycorubins obtained with $NaBH_4$ as true reduction products, their properties are quite different. The major difference between the addition of nucleophiles to and the hydrogenation of biliverdin is, that the former reaction is in thermodynamic equilibrium, whereas the latter is essentially irreversible under the reaction conditions. Biliverdin can be reduced to bilirubin only with rather strong reductants (*e.g.* $NaBH_4$), whereas the oxidation of bilirubin to biliverdin requires high-potential quinones* [4, 14]. Recent electrochemical studies have shown, too, that the biliverdin-bilirubin redox couple is not an equilibrium system [26]. The striking difference between phycorubin and the addition products of phycocyanin summarized in Scheme 1 can readily be explained on this basis. The yellow phycorubin chromophore remains stable upon renaturation of the protein and removal of the reducing agent, sodium borohydride. Moreover, the absorption maximum remained roughly in the same position and the extinction coefficient was the same within experimental error. The geometry of the phycocyanin chromophores is profoundly changed when the protein is renatured and *vice versa*, which is reflected by pronounced spectral changes [25]. The absence of similar effects in phycorubin could then indicate, that the altered chromophores do no longer fit the binding sites, and remain – although covalently attached – only loosely coupled to the protein. It should be noted, though, that the uv-vis-spectra of bilirubins [28] appear less conformation dependent than those of biliverdins [29].

The different binding situation of the chromophores in native phycocyanin and phycorubin can also be the reason for their different electrophoretic mobilities (Fig. 2). Phycorubin migrates faster towards the anode, which could be due to an increase of the net negative charge by the free carboxylic acid side chain(s) at or close to the surface. The ready reaction of renatured phycorubin with diazotized ethyl anthranilate leading to a product which cannot be distinguished uv-vis spectroscopically ($\lambda_{max} = 485$ nm) from the denatured form, is compatible with this proposal. In SDS-gel electrophoresis, PC and phycorubin migrated identically, indicating the absence of proteolytic artefacts during the preparation of the latter pigment.

In view of the irreversible reactions of $NaBH_4$, the treatment of native PC may be used to discriminate between thermodynamic and kinetic effects in the reactions of biliprotein chromophores. In first order, one may assume that (i) the chromophores react with $NaBH_4$ if only their methine bridges are accessible to the reagent, and that (ii) this reaction may be slowed down by the influence of the protein, but is irreversible once the chromophore has been reduced. The influence of the state of the protein on the reaction kinetics with $NaBH_4$ has already been studied by Crespi *et al.* [45], and interpreted as a change in accessibility of the cromophores to the reagent. An alternative mechanism of the protein to slow down the reaction with sodium borohydride would be a conformational change of the chromophore. The similar reactivity of biliverdin and isophorcabilin does not prove this assumption, but indicates that a mere conformational change without the coplanar fixation suggested for PC has no dominant influence.

The reaction of native PC with dithionite is incomplete even at high concentrations of the reagent. In this case the reaction is determined by thermodynamics. Sodium borohydride reduces native PC slowly, but completely. This demonstrates, that C-10 of all chromophores is accessible by the reagent dissolved in the aqueous phase. Similar conclusions have been reached by Troxler [30] for the terminal O-1 and O-19, and hence practically the entire chromophore must be accessible to the aqueous phase. It requires further work to determine, whether the chromophores are actually at the

* We have achieved the reoxidation of phycorubin with benzoquinone to products identical with PC if judged from absorption, fluorescence and electrophoretical mobilities. Details of this procedure and the reaction of PC with quinones [27] are to be published separately.

surface, or rather in interior hydrophilic parts of the protein.

The possibility of the preparation of a phycobiliprotein with a rubin-type chromophore described here makes further modifications feasible, since bilirubins are accessible to a variety of reactions. A first example is the use of the diazoreaction of bilirubin as a novel degradation technique for biliproteins [31].

## Materials and Methods

PC was isolated from *Spirulina platensis* as described previously [3].

### *Preparation of Phycorubin*

10.0 ml of a solution of denatured PC ($5 \times 10^{-5}$ M chromophores) in 50 mM sodium phosphate/8 M urea-buffer (containing 5 mM EDTA and 5 mM $NaN_3$), pH 7.5, were cooled with ice and 500 µl of a solution of 10 mg/ml sodium borohydride in the same buffer were added. The solution was left standing for 1/2 h and then again 500 µl of the borohydride solution were added ($2.4 \times 10^{-2}$ M final concentration). Within again 1/2 h the reaction followed spectrophotometrically went to completion.

### *Renaturation of phycorubin*

For removal of urea (and borohydride) 1.0 ml of the solution obtained by the reaction described above was passed through a 1.7 × 12 cm biogel P 2 column (Bio-Rad, Richmond, California) which was previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.5. The procedure was carried out in a cold room at 4 °C.

### *Preparation of model bilins for the PC-chromophore and of authentic rubins*

Bilirubin (**4a**), biochemical grade, was purchased from Merck, Darmstadt. Mesobilirubin (**4b**) was prepared from 200 mg of **4a** by catalytic hydrogenation [4, 32] (40 mg of 10% Pd on charcoal) in 20 ml 0.1 N NaOH. The reaction was followed spectrophotometrically, adding aliquots from the reaction mixture to methanol ($\lambda_{max}$ (**4a**) = 452 nm, $\lambda_{max}$ (**4b**) = 428 nm). The mixture was worked up with chloroform after addition of glycine/HCl-buffer, pH 2.7, saturated with ammonium sulfate. Yield: 98%. Biliverdin (**3a**) was prepared from 50 mg bilirubin (**4a**) dissolved in 50 ml dimethylsulfoxide by oxidation with a solution of 50 mg 2,3-dichloro-5,6-dicyanobenzoquinone in 10 ml of the same solvent in a nitrogen atmosphere [4]. After work-up with chloroform and water (yield: 38%), the reaction product was purified by preparative tlc on silica using the upper phase of a mixture of toluene/acetic acid/water 5:5:1 (v/v/v) as solvent system. The green band ($R_F$-value 0.1) was eluted with acetic acid and worked up by partitioning between chloroform and water. The chloroform phase was washed with water and 1% aqueous $NaHCO_3$-solution until neutral (yield: 26%). For crystallization the product was dissolved in 3 ml chloroform (containing a small amount of methanol). 12 ml of *n*-hexane were added and the mixture was left standing at − 20 °C over night (yield: 9%).

Mesobiliverdin (**3b**) was prepared by an analogous procedure from mesobilirubin (**4b**).

10 mg of crude (**3a**) dissovled in 150 ml methanol were esterified to the corresponding dimethylester (**3c**) by treatment with 60 ml methanol containing 20% (w/w) $BF_3$ [4] under reflux for 10 min in a nitrogen atmosphere. After work-up with chloroform and water, the crude product was purified by tlc on silica with chloroform/acetone 95:5 (v/v) as solvent [33]. The green zone was eluted with acetone (yield: 85%).

Bilirubin dimethylester (**4c**) was prepared from 30 mg (**4a**) suspended in 10 ml $CHCl_3$ by esterification with diazomethane over night [34, 35]. The chloroform phase was washed with an aqueous 10% $Na_2CO_3$ solution and the product subsequently chromatographed on neutral $Al_2O_3$ (2 × 6 cm; super activity I, Woelm, Eschwege) [36]. By-products were eluted with $CHCl_3$, (**4c**) with $CHCl_3$/MeOH 9:1 (yield: 65%).

Octaethylbilindiones (**7**) and (**8**) were synthesized from octaethylporphyrin [37, 38]. The products were purified by tlc on silica with $CCl_4$/acetone 9:1 (v/v) as solvent.

Biliverdin-IX$\gamma$-dimethylester (**11**): a mixture of the isomeric biliverdin-IX dimethylesters was obtained by coupled oxidation ($O_2$/hydrazine) of hemin with subsequent esterification [33]. Biliverdin-IX dimethylester was freed from the $\beta$ and $\delta$-isomers by tlc on silica with chloroform/acetone 97:3 (v/v) [39], and subsequently from the $\alpha$-isomer by tlc on silica with toluene/ethylmethylketone/acetic acid 10:5:0.5 (v/v/v) [40].

Phorcabilindimethylester (**9**) was prepared by heating biliverdin-IX $\gamma$-dimethylester (**11**) for 1 h at 100 °C in dimethylsulfoxide under nitrogen [39]. Isophorcabilindimethylester (**10**) was obtained from phorcabilindimethylester (**9**) in 20% MeOH/$H_2SO_4$, the mixture being kept under reflux for 1/2 h [39]. The products were purified by tlc on silica with chloroform/acetone 8:2 (v/v) as solvent system and crystallized from $CHCl_3$/*n*-pentane 1:25 at − 20 °C.

Mixture of the III-, IX- and XIII-$\alpha$-isomers of bilirubin (**4a**, **5a**, **6a**) and mesobilirubin (**4b**, **5b**, **6b**): bilirubin (**4a**) or mesobilirubin (**4b**) were dissolved in 0.5 ml 0.1 N NaOH and added to 5.5 ml 50 mM sodium phosphate buffer, pH 7.5, containing 8 M urea. The samples were purged with nitrogen and kept at 34−38 °C for 2 h. The rubins were extracted with chloroform after addition of glycine/HCl buffer, pH 2.7, saturated with $(NH_4)_2SO_4$. For preparation of pure isomers of bilirubin, the mixture was chromatographed on silica with $CHCl_3$/HOAc 97:3 (v/v) [7c] and the products eluted with chloroform.

The model compounds were reacted with sodium borohydride (Merck-Schuchardt, Hohenbrunn, p.s.) and sodium dithionite (technical grade, Merck, Darmstadt). The reaction mixtures were worked up by extraction with 1.5 vol of chloroform after addition of 1.5 vol. glycine/HCl-buffer, pH 2.7, saturated with ammonium sulfate in the case of free acids [5]. The chloroform phase was briefly dried over NaCl and after filtration on cotton, uv-vis spectra were taken. The samples were dried in a stream of nitrogen for tlc.

*Preparative scale reaction of biliverdin dimethylester* (**3c**) *with dithionite*

A solution of 10 mg **3c** in 10 ml methanol was added to a solution of 50 mg sodium dithionite in 10 ml doubly glass distilled water under a nitrogen atmosphere. After 10 min, 20 ml glycine/HCl-buffer, pH 2.7 [5] were added and nitrogen was bubbled through the solution for further 30 min. The mixture was extracted twice with 10 ml chloroform, with some yellow pigment remaining in the aqueous phase. Preparative tlc of the organic extracts on silica gel (0.75 mm) with benzene/ethanol = 25:2 (v/v) yielded four zones: 1, yellow, $R_F = 0$; II, yellow, $R_F = 0.3-0.5$; III, yellow with greenish and brownish components, $R_F = 0.65$; IV, blue, $R_F = 0.70$ (identified as the starting material by analytical tlc with system "a" of Table I). Bands II−IV were eluted with acetone, elution of zone I was impossible. Zones II and III were each fractionated by column chromatography (2 × 5 cm valumina, neutral, activity super 1; Woelm, Eschwege): zone II: the main fraction was eluted with chloroform, a minor fraction with chloroform/methanol 9:1, zone III: two fractions were separated with chloroform, one with chloroform/methanol 9:1, a further zone could not be eluted with methanol.

*Preparative scale reaction of biliverdin* (**3a**) *with dithionite*

7.8 mg biliverdin were dissovled in 50 ml hot methanol and after cooling, added to a solution of 500 mg sodium dithionite in 50 ml water under a continous stream of nitrogen. 400 ml glycine/HCl-buffer, saturated with ammonium sulfate, pH 2.7 [5], were immediately added and the aqueous phase was extracted four times with a total volume of 300 ml of chloroform. The chloroform phase was concentrated in vacuo to 30 ml and fractionated by tlc on silica gel plates (0.25 mm) with benzene/ethanol = 25:2 (v/v). A zone with green, brown and violet coloured substances with a yellow margin remained at the start. The only major zone (orange $R_F = 0.4$) was eluted with 130 ml chloroform to yield 0.8 mg crude bilirubin (**4a**); it was esterified with $CH_2N_2$ in $CHCl_3$. Chromatography on $Al_2O_3$ yielded the following fractions: I, yellow substance eluted with chloroform; II, main fraction, yellow substance eluted with $CHCl_3$/MeOH 9:1 = yield 0.3 mg; $\lambda_{max}$ ($CHCl_3$) = 403 nm, shoulder at 435 nm; identical in cochromatography with bilirubin-dimethylester (**4c**) with system a of Table I; III, yellow-greennish substance, eluted with $CHCl_3$ acetic acid = 1:1.

$^1$HMR-spectra of biliverdin dimethylester (**3c**), its reaction products with borohydride and dithionite, and of authentic bilirubin dimethylester (**4c**):

Preparation of the reactions products: **3c**/$NaBH_4$ and **3c**/dithionite: A solution of 6 mg biliverdin dimethylester (**3c**) in 2 ml $CHCl_3$ was shaken with 1.0 ml of an aqueous solution of 50 mg/ml dithionite until the colour changed to yellow-green. The $CHCl_3$ phase was washed with water, dried on NaCl and evaporated under a stream of nitrogen. An analogous experiment was carried out using sodium borohydride instead of dithionite.

Biliverdin dimethylester (**3 c**): 1.80 (exo-$CH_3$); 2.01, 2.04, 2.10 (*endo*-$CH_3$; 2.56 t, 2.93 t (8.12-$CH_2$-$CH_2$); 3.61 ($OCH_3$); 5.28–6.61 (vinyl-protons); 5.94, 6.00 (5.15-CH); 6.73 (10-CH).

Bilirubin dimethylester (**4 c**): 1.68 (*exo*-$CH_3$); 1.91, 202 (*endo*-CH; 2.39 t, 2.84 t (8.12-$CH_2$-$CH_2$); 3.63 ($OCH_3$); 4.10 (10-$CH_2$); 4.65–6.66 (vinyl protons); 5.84, 613 (5.15-CH); 10.04, 10.12, 10.42, 11.14 (NH).

**3 c**/$NaBH_4$: 1.68 (*exo*-$CH_3$); 1.91, 201 (*endo*-$CH_3$); 2.38 t, 2.83 t (8.12-$CH_2$-$CH_2$); 3.63 ($OCH_3$); 4.09 (10-$CH_2$); 4.65–6.66 (vinyl protons); 5.84, 6.12 (5.15-CH); 10.03, 10.12, 10.41, 11.11 (NH).

**3 c**/dithionite: Broad signals in the ranges 1.5–3.6 ppm and 5.1–6.4 ppm; no signal detectable in the range 3.7–5.0 ppm.

*General methods:*

UV-vis spectra were taken with a model DMR 22 spectrophotometer (Zeiss, Oberkochen), the spectra of compounds 9–12 with a model Superscan spectrophotometer (Varian, Palo Alto). $^1$Hmr spectra were recorded in C $^2HCl_3$ with a model HFX 90 spectrometer (Bruker, Karlsruhe). Chemical shifts in $\delta$ (ppm) are given relative to $Si(CH_3)_4$ as internal standard. Signals appeared as singlets, unless otherwise noted. t = triplet. The vinyl protons appeared as ABX system.

Tris-glycine electrophoresis was done by the method of Davies [41] as modified by Wagenmann [42], SDS-electrophoresis with the system of Weber and Osborn [6]. Densitograms were obtained with a type TLD 100 densitometer (Vitatron). Analytical tlc was carried out on silica HPTLC plates and polyamide 11 F 254 plates (both Merck, Darmstadt). Preparative separations were performed on self-made 20 × 20 cm plates covered with 0.75 mm silica H (Merck, Darmstadt).

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